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PATENT & TRADEMARK OFFICE

PATENT
Attorney Docket No 03804-8050-00000

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicant(s):	Bouchard et al.	Group Art Unit:	1206
Serial No	08/162,984	Examiner:	J Peabody
Filed	December 8, 1993		
For:	NEW TAXOIDS, THEIR PREPARATION AND PHARMACEUTICAL COMPOSITION CONTAINING THEM		

DECLARATION OF FRANCOIS LAVELLE

Honorable Commissioner of Patents & Trademarks
Washington, D.C. 20231

Sir:

I, FRANÇOIS LAVELLE, make the following declaration:

1. I am the Director of the Department of Biologie, Service de Cancérologie by RHÔNE-POULENC RORER RECHERCHE-DEVELOPPEMENT, the wholly owned subsidiary of RHÔNE-POULENC RORER S.A., the assignee of the above-identified application (the "'984 Application").

2.

2a. I received a Doctorat ès Sciences at the Université de Paris. I have been employed in the position of Director of the Department of Biologie, Service de Cancérologie for 17 years. Included in my responsibilities is the supervision of biological assays of compounds for anti-tumor activity and in particular the assay of compounds in the taxoid family for properties of tumor cell growth inhibition and tumor cell death. I am a co-author on numerous publications including those listed in attached Appendix I.

2b. Based upon my professional and educational background and experience, I am familiar with anti-tumor compounds, including compounds of the taxoid family, and their pharmacological profiles, including their anti-tumor properties. In this declaration I will present and explain the results of studies comparing the anti-tumor properties of three members of the taxoid family: a cyclopropyl taxoid compound referred to herein as Compound I, and two other cyclopropyl taxoid compounds, referred to herein as Compounds II and III, which are the closest structural analogues of Compound I disclosed in the 08/162,984 patent application ('984 application) and U.S. Patent No. 5,254,580 (10/19/93) to Chen et al., assigned to Bristol-Myers Squibb Company (the "'580 patent").

2c. I executed a declaration on December 27, 1994, that was filed in this application on December 29, 1994. Since that time, I have had the occasion to further study that declaration and found some inadvertent errors. To correct those errors, to expand on and

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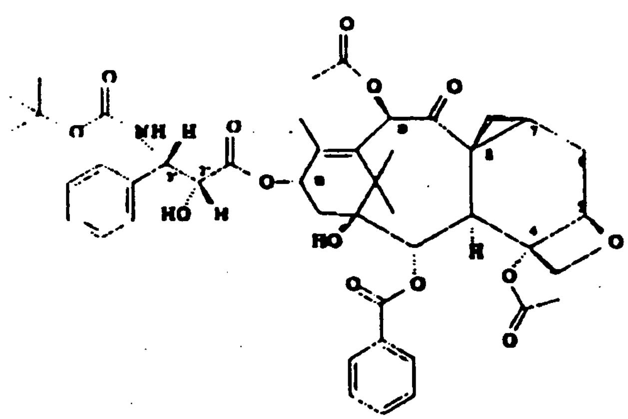
clarify other points, and to omit certain information which I understand to be irrelevant. I am withdrawing my previous declaration and present this new replacement declaration for consideration by the United States Patent and Trademark Office.

3. I organized and directly supervised the pharmacological study of Compounds I, II, and III Specifically, I supervised biological studies which compared the anti-tumor properties of a formulation containing Compound I with those of otherwise identical formulations respectively containing Compounds II and III.

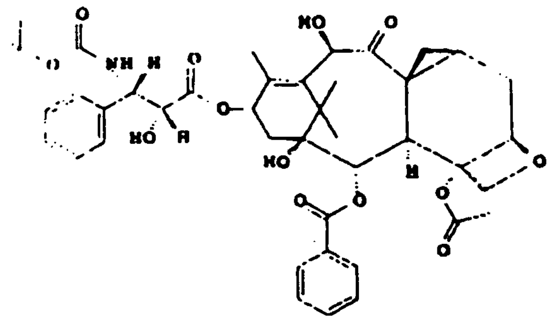
4 Compound I, which was studied, can be named 4α-10ß-diacetoxy-2α-benzoyloxy-5ß,20-epoxy-1ß-hydroxy-7ß,8ß-methylene-9-oxo-19-nor-11-taxen-13α-yl (2R,3S)-3-tert-butoxycarbonylamino-2-hydroxy-3-phenylpropionate, which is exemplified in the '984 application in Example at pp 40-41. Compound I is also disclosed in Example 23 of the '580 patent. The '580 patent identifies Compound I by the name of N-debenzoyl-N-t-butoxycarbonyl-7-deoxy-8-desmethyl-7,8-cyclopropataxol, which is synonymous with the name given for Compound I in the '984 application.

5. Compounds II and III are exemplified in the '984 application in Example 1, p. 30 (Compound II), in Example 2 of the '984 application at pp. 36-37 (Compound III), and in the '580 patent in Examples 3 and 21 (Compound III). Compound II also falls within the genus of formula I found at column 1 of the '580 patent but is not exemplified in the '580 patent

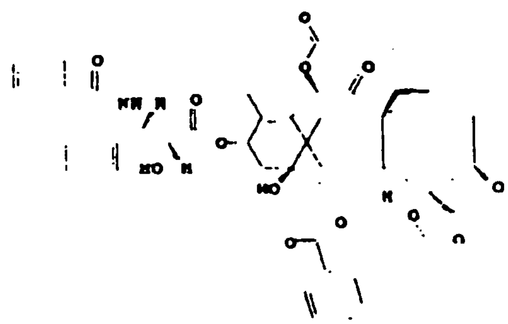
6 Compounds I, II, and III have the following structural formulae:



COMPOUND I



COMPOUND II



COMPOUND III

7. Compound II differs from Compound I only in that Compound II has an hydroxy group instead of an acetoxy group at position 10. Compound III differs from Compound I only in that instead of the t-butoxy group on the side-chain, Compound III has a phenyl group.

8.

8a. The biological studies which I supervised compared the in vitro and in vivo anti-tumor properties of Compounds I, II and III. The purpose of the in vivo test was to assess relative antitumor activity.

8b In the in vitro study, the anti-tumor properties of compounds I, II, and III were compared against two different tumor cell lines characterized by low and high expression of the multi-drug resistance gene. It is known that some cancers, such as colon cancer, are intrinsically drug resistant while others acquire resistance following chemotherapy.

This phenomenon is known in the art as multidrug resistance, and multi-drug resistant cell lines usually contain an amplified gene, termed MDR1 in the human Thus, in the treatment of such types of cancer, the multidrug resistance properties of a drug are highly significant. In general, see J Natl Cancer Inst 1989;81:116-124, attached as Appendix II.

<u>Description of the In Vitro Test</u>

8c The <u>in vitro</u> activity is evaluated in the P388 murine leukemia cell line and the P388 murine leukemia cell line resistant to doxorubicin and expressing the multi-drug resistance gene (P388/DOX). Use of these murine leukemia cell lines in evaluating multi-drug resistance is well-accepted in the art, as exemplified in Cancer Treat Rep 67:905-922, 1983, attached as Appendix III.

8d. 3×10^5 cells/ml were grown for 96 hours in the presence of various drug concentrations. Cells were then incubated for 16 hours with 0.02% natural red, washed and lyzed with 1% SDS (sodium dodecyl sulphate).

8e. The incorporation of the dye reflecting the cellular growth was assayed by optical density measurement at 540 and 346 nm.

8f The concentration of the drugs resulting in 50% growth inhibition (IC_{50}) was then determined: the lower the IC_{50} value the higher the potency of the compound.

8g. The lower the ratio (IC_{50}-P388/DOX)/(IC_{50}-P388), the "Resistance Factor R," the higher the activity of the compound as an effective tumor cell growth inhibitor of multi-drug resistant cell lines.

8h. The results of the comparative <u>in vitro</u> study are presented in the following Table A.

TABLE A

Compound	IC_{50} (µg/ml) P388	IC_{50}(µg/ml) P388/DOX	Resistance Factor R
I	0.03	0.25	8
II	0.03	0.45	15
III	0.085	1.80	21

Description of the In Vivo Test

8i In the in vivo study, antitumor activity of compounds I, II and III were evaluated in B16 melanoma-bearing mice wherein tumors were implanted as subcutaneous bilateral fragments in B6 D2F1 mice.

Description of the methodology

8j The animals necessary to begin a given experiment were pooled and implanted-subcutaneously bilaterally with 30 to 60 mg tumor fragment on day 0 with a 12 gauge trocar. Bilateral implants were used to insure a more uniform burden per mouse and thus reduce the requirement for a greater number of mice per group.

8k. For an early stage tumor treatment, the tumor-bearing animals were again pooled before unselected distribution to the various treatment and control groups.

8l. For an advanced stage treatment, the solid tumors were allowed to grow to the desired size range (animals with tumors not in the desired range were excluded). The mice were then pooled and unselectively distributed to the various treatment and control groups.

8m. Non tumor bearing animals (NTBA) were often matched to tumor-bearing groups and given the same route, dose and schedules. In this way, drug-induced toxicity can be clearly separated from the effects of the tumors.

8n. Chemotherapy was started within 3 to 24 days after tumor implantation. Compounds I, II, and III were injected intravenously (i.v.) under a volume of 20 ml/kg. Mice were checked daily and adverse clinical reactions were noted.

8o. Each group of mice was weighed as a whole three to five times weekly until the weight nadir was reached. The groups were weighed once or twice weekly until the end of the experiment.

8p Tumors were measured with a caliper twice or three times weekly until the tumors reached 2,000 mg or until the animal died (whichever came first).

8q. Solid tumor weights were estimated from two dimensional tumor measurements

$$\text{Tumor weight (mg)} = \frac{\text{length (mm) x width 2 (mm}^2\text{)}}{2}$$

8r. The day of death was recorded. Surviving animals were killed and macroscopic examination of the thoracic and abdominal cavities was carried out. In some cases, tissue samples were submitted to histological evaluation.

- End point for assessing antitumor activity

8s Antitumor activity evaluation was done at the highest non-toxic dosage (HNTD). "HNTD" is defined as the dose which gives no lethality and produces less than 20% body weight loss at nadir. A dosage producing 20% weight loss nadir (mean group) or 20% or more drug deaths, was considered an excessively toxic dosage. Animal body weights included tumor weights.

- Tumor growth inhibition (T/C)

8t. The treatment and control groups were measured when the median of the control group tumors reached approximately 750 to 1,200 mg. The median tumor weight of each group was determined.

8u. The T/C value in percent is an indication of antitumor effectiveness.

$$T/C\ (\%) = 100 \times \frac{\text{median tumor weight of the treated groups}}{\text{median tumor weight of the control groups}}$$

8v According to NCI (National Cancer Institute) Standards, a T/C $\leq$ 42% (score +) is the minimal level to declare activity. A T/C < 10% (score: ++) is considered to indicate high anti tumor activity and is the level used by NCI to justify further development. This is indicated in Instruction 271B, dated April 1, 1978, attached as Appendix IV. As is seen therein, there are four types of tumors for which median tumor weight is the appropriate parameter, as in the tests described herein. In all four instances, the "Initial Activity" is reported at $\leq$42. In the three instances where further studies were reported, DN2 is given as $\leq$10%. DN2 means decision number 2, thus reflecting a level that would justify further development, according to the National Cancer Institute standards.

- Tumor growth delay

8w T and C are the median times (in days) required for the treatment group and the control group tumors respectively to reach predetermined size (usually 750 to 1,000 mg) Tumor free survivors are excluded from these calculations and tabulated separately.

8x. This value is the more significant one as it allows the quantification of the tumor cell kill, discussed below as log cell kill.

- Determination of the tumor doubling time (Td)

8y. Td is estimated from the best fit straight line from a log linear growth plot of the control group tumors in exponential growth (100 to 1,000 mg range).

- Quantification of tumor cell kill

8z. For subcutaneous growing tumors, the total log cell kill is calculated from the following formula:

$$\text{log cell kill (gross or total)} = \frac{\underline{(T - C)\ \text{value in days}}}{3.32 \times Td}$$

where T-C is the tumor growth as described above and Td is the tumor volume doubling time in days.

8aa. The log cell kill value can be converted to an arbitrary activity rating with the following table as is shown at page 718 of CANCER RESEARCH 44, 717-726, February 1984, attached as Appendix V:

Activity	Duration of treatment (5-20 days) $\log_{10}$ kill gross
Highly active ++++	> 2.8
+++	2.0 to 2.8
++	1.3 to 1.9
+	0.7 to 1.2
Inactive -	< 0.7

8ab. With respect to log cell kill value, there is a significant difference between ratings of +++ and ++++ as compared to + and ++. This is explained at page 718 of Appendix V as follows

> An activity rating of +++ or ++++ is needed to effect partial or complete regression of 100- to 300-mg masses of most transplanted solid tumors of mice. Thus, an activity

rating of + or ++ would not be scored as active by usual clinical criteria. (footnote omitted)

8ac. The results of the comparative in vivo study are presented in the following Table B. I have included data relating to an additional test regarding Compound I that was overlooked when my December 1994 declaration was prepared. Also, the box below Table B has been modified to correct an error in transcription regarding the log cell kill scores that occurred in my December 1994 declaration and to add some additional clarifying information regarding the T/C x 100 scores. These changes are consistent with the information given in Appendices IV and V.

TABLE B

Compound	T/C x 100	Score	Log cell kill	Score
I	6	++	2.7	+++
I	16	+	2.0	+++
II	17	+	1.0	+
III	53	-	not relevant	not relevant

In the experiments: tumor (B16 melanoma) grafted s.c. on day 0 in mice; i.v. treatment on days 5, 7 and 9.

Score (T/C x 100): T/C < 10 : ++ (highly active); T/C from 10 to 42: + (active); T/C > 42: - (inactive) (see Appendix IV).

Score (Log cell kill); < 0.7 : - ; from 0.7 to 1.2: + ; from 1.3 to 1.9: ++ , from 2.0 to 2.8: +++ (see Appendix V).

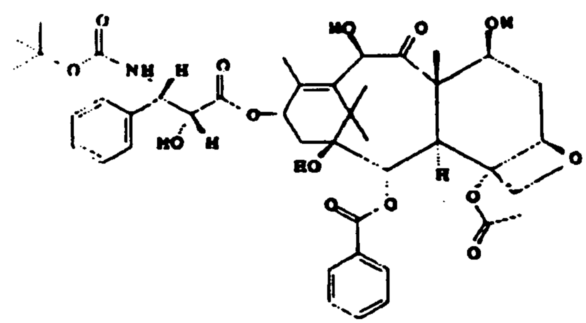
8ad. Based on my experience and education, "log cell kill" is more closely related to tumor regulation than is "T/C x 100", which is consistent with the fact that Appendix V refers only to "log cell kill" and not to "T/C x 100" with respect to antitumor activity. Further, I note that since it was determined that Compound III is inactive in accord with the NCI T/C standards set forth in Appendix IV, "log cell kill" for Compound III is irrelevant and was not evaluated.

CONCLUSION

9. Based upon the results of the biological evaluation shown in the above Tables A and B, it is my professional opinion that Compound I is the superior anti-tumor compound in comparison to the closely related compounds II and III.

9a As shown by the in vitro tests, Compound I significantly has about 2-3 fold more effective multi-drug resistance properties than Compounds II and III.

9b. In the in vivo tests, Compound I, having a log cell kill arbitrary activity rating (see the Table above in ¶ aa) of +++ , was superior to Compound II, which, although active, demonstrated a log cell kill arbitrary activity rating of only + . Even though the two tests run on Compound I had log cell kills that differed by 0 7, the important point is that both values correspond to an arbitrary activity rating score of +++ As part of my experience explained above, I have had the occasion to do many in vivo tests of the same type described above on the known TAXOTERE® antitumor compound , which is also a member of the taxoid family and has the following structural formula:



TAXOTERE® Antitumor Compound

To the best of my recollection, even though the log cell kill values of TAXOTERE® antitumor compound have differed in numerical value in these in vivo tests, the arbitrary activity rating score has always been +++ . Thus, I have no reason to believe that if I repeated the in vivo test for Compound II, I would obtain a different arbitrary activity rating score.

9c. The in vivo tests also demonstrate that, in accord with the T/C x 100 evaluations, Compound I is active, and Compound III is inactive. Such a difference between active and inactive is significant, even though the log cell kill is more closely related to tumor regulation.

9d. Thus, in view of the close structural similarities of Compounds I, II, and III, I consider that the multi-drug resistance properties and the log cell kill properties reported herein, taken together, demonstrate that Compound I is unexpectedly superior to Compounds II and III

10 I declare further that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true and further that these statements were made with the knowledge that willful false statements and the like so

made are punishable by fine or imprisonment, or both, under Section 1011 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the '984 Application or any patent issuing thereon.

Dated. April 14, 1995

By: François Lavelle
François Lavelle

lavel74.dcc

APPENDIX I

APPENDIX I

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APPENDIX II

APPENDIX II

ARTICLES

Expression of a Multidrug Resistance Gene in Human Cancers

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Many cancers have been cured by chemotherapeutic agents. However, other cancers are intrinsically drug resistant, and some acquire resistance following chemotherapy. Cloning of the cDNA for the human MDR1 gene (also known as PGY1), which encodes the multidrug efflux protein P-glycoprotein, has made it possible to measure levels of MDR1 RNA in human cancers. We report the levels of MDR1 RNA in >400 human cancers. MDR1 RNA levels were usually elevated in untreated, intrinsically drug-resistant tumors, including those derived from the colon, kidney, adrenal gland, liver, and pancreas, as well as in carcinoid tumors, chronic myelogenous leukemia in blast crisis, and cell lines of non-small cell carcinoma of the lung (NSCLC) with neuroendocrine properties. MDR1 RNA levels were occasionally elevated in other untreated cancers, including neuroblastoma, acute lymphocytic leukemia (ALL) in adults, acute nonlymphocytic leukemia (ANLL) in adults, and indolent non-Hodgkin's lymphoma. MDR1 RNA levels were also increased in some cancers at relapse after chemotherapy, including ALL, ANLL, breast cancer, neuroblastoma, pheochromocytoma, and nodular, poorly differentiated lymphoma. Many types of drug-sensitive and drug-resistant tumors, including NSCLC and melanoma, contained undetectable or low levels of MDR1 RNA. The consistent association of MDR1 expression with several intrinsically resistant cancers and the increased expression of the MDR1 gene in certain cancers with acquired drug resistance indicate that the MDR1 gene contributes to multidrug resistance in many human cancers. Thus, evaluation of MDR1 gene expression may prove to be a valuable tool in the identification of individuals whose cancers are resistant to specific agents. The information may be useful in designing or altering chemotherapeutic protocols in these patients. [J Natl Cancer Inst 1989;81:116-124]

Chemotherapeutic agents have proven to be effective in the cure or palliation of some human cancers; however, both intrinsic drug resistance and acquired drug resistance remain clinical obstacles in the treatment of many other cancers. For the study of the mechanisms of multidrug resistance, tumor cell lines have been selected for resistance to the *Vinca* alkaloids, doxorubicin, dactinomycin, and related natural products (*1-5*). Intracellular drug accumulation has been found to be decreased secondary to increased drug efflux in these cell lines (*2,6*). These multidrug-resistant cell lines usually contain an amplified gene, termed MDR1 (also known as PGY1) in the human, that is transcribed into a 4.5-kilobase mRNA (*7-12*). The protein product of this gene is a 170-kilodalton

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membrane glycoprotein, called P-glycoprotein or the multidrug transporter, wh- is an energy-dependent drug efflux pump (*13,14*).

A full-length cDNA for the MDR1 gene from one of the multidrug-resistant human KB carcinoma cell lines has been isolated and sequenced (*15,16*). With the use of a region of this cDNA as a probe, the MDR1 gene has been shown to be expressed at a high level in normal human kidney, adrenal gland, liver, and colon (*17*). In the kidney, liver, and colon, the MDR1 gene product (P-glycoprotein) was present on the luminal surface of epithelial cells, which is consistent with a normal role of this protein as a transporter (*18*). In addition, several human cancers, including adenocarcinomas derived from tissues that normally express the MDR1 gene, have been shown to overexpress MDR1 RNA (*17,19*). Immunochemical analysis revealed overexpression of P-glycoprotein in two of five patients with ovarian carcinoma (*20*) and in two patients with drug-resistant acute nonlymphocytic leukemia (ANLL) (*21*). In 25 patients with sarcoma, six tumor samples had elevated levels of P-glycoprotein (*22*).

To investigate further the association of the expression of the MDR1 gene in human cancers with drug resistance, we have measured MDR1 RNA levels in many types of human cancers. We report here measurements of MDR1 RNA levels in >400 human cancer specimens. Our results identify four groups of cancers: (*a*) cancers that usually express high levels of MDR1 RNA, (*b*) cancers that occasionally express high levels, (*c*) cancers that rarely express MDR1 RNA, and (*d*) cancers that express the MDR1 gene at elevated levels after exposure to chemotherapeutic agents. Taken together, these results are consistent with an important role for the MDR1 gene in clinical drug resistance and suggest that measurements of MDR1 RNA can be useful in the design of chemotherapeutic protocols for certain tumors.

Materials and Methods

Cell Lines

KB-3-1 is the drug-sensitive parental KB (HeLa) cell line. KB-8-5, which is four times as resistant to doxorubicin and six times as resistant to vinblastine, was derived in two steps from KB-3-1 by selection in colchicine (*4*). KB-8-5 has increased levels of MDR1 mRNA without gene amplification (*7*). Cell line KB-C1 was derived in two further steps from KB-8-5 and is 160 times more resistant to doxorubicin and 96 times more resistant to vinblastine than KB-3-1 is (*6*). It has amplified the MDR1 gene about 100-fold and expresses MDR1 mRNA at a very high level (*7*).

MDR1 Hybridization Probes

cDNA was prepared with the use of RNA from KB-C2.5 cells, which contain large amounts of MDR1 mRNA, and was inserted into the EcoRI site of bacteriophage λgt11 (*15*). Probe 5A, which encodes about one-third of the coding region of a full-length MDR1 cDNA, was labeled by nick translation before use in the RNA slot blot analyses (*15*). An MDR1 genomic fragment of 785 base pairs (bp) that was derived from PvuII-digested plasmid pMDR-P2 was used to make a riboprobe with SP6 polymerase for the RNase protection assays. This fragment contains the transcription-initiation sites of the downstream promoter and additional sequences 5' to the downstream promoter (*23*). Deoxycytidine 5'-[α-^{32}P]triphosphate (3,000 Ci/mmol; Ci = 37 GBq) and uridine 5'-[α-^{32}P]triphosphate (3,000 Ci/mmol) were from DuPont/NEN Products (Boston, MA). Promega Biotec (Madison, WI) was the source of the PGEM4 and the Riboprobe Gemini System II. The Amersham Corporation (Arlington Heights, IL) manufactured the nick-translation system.

RNA Extraction and Electrophoresis

All samples were stored frozen at −70 °C. Before RNA extraction, solid tumors were pulverized on a metal surface on a bed of dry ice. Buffy coats from leukemia samples or leukemia blast cells isolated by Ficoll–Hypaque gradient centrifugation and frozen in 10% dimethyl sulfoxide were thawed rapidly at 37 °C and centrifuged. For lung cancer and mesothelioma, cell lines were available for analysis. The lung cancer cell lines were established, grown, and characterized as previously described (*24-28*). Tissue culture dishes and flasks of cell lines were washed twice with phosphate-buffered saline without calcium and magnesium. Total cellular RNA was extracted by homogenization in guanidinium isothiocyanate followed by centrifugation over a cesium chloride cushion (*29*) or by acid-phenol extraction (*30*). The RNA was electrophoresed in 1% agarose–6% formaldehyde gels. One microgram of total RNA was loaded per lane. The ribosomal RNA appeared undegraded in almost all samples reported here. Samples with degraded RNA were not further analyzed.

Slot Blot Analysis

Nitrocellulose filters were presoaked in 10× SSC (1× SSC = 0.15 *M* NaCl/15 m*M* sodium citrate, pH 7). Serial dilutions of 10, 3, 1, and 0.3 µg of each sample of total RNA in 10× SSC were applied to each well of a Schleicher and Schuell slot blot apparatus. After baking at 80 °C in a vacuum oven, the filters were prehybridized for 4-6 hours at 42 °C in 50% formamide, 5× SSC, 5× Denhardt's solution (1× Denhart's solution = 0.02% Ficoll, 0.02% polyvinylpyrrolidone, and 0.02% acetylated bovine serum albumin), 50 m*M* sodium phosphate (pH 6.5), and 200 µg of salmon sperm DNA/mL. The filters were then hybridized for 16 hours at 42 °C in 50% formamide, 5× SSC, 1× Denhardt's solution, 10% dextran sulfate, 100 µg of salmon sperm DNA/mL, and 20 m*M* sodium phosphate (pH 6.5) with 5 × 10^6 cpm of nick-translated cDNA/mL. After hybridization, the filters were washed four times for a total of 1 hour with 1× SSC/0.1% sodium dodecyl sulfate (SDS) at 23 °C followed by two 10-minute washes with 0.2× SSC/0.1% SDS at 50 °C. Autoradiographs were exposed for 1-5 days. Hybridization with a nick-translated γ-actin probe (*31*) was performed to compare RNA loading.

RNase Protection Assay

The starting sites of MDR1 transcription in various human cell lines and tumors were mapped with an RNase protection

assay with the use of a labeled SP6 anti-sense RNA probe (785 nucleotides) deri--d from the PvuII-digested plasmid described above. Tw micrograms of total RNA from each sample was hybridized with 3×10^5 cpm of the riboprobe, and RNase digestion was performed as previously described (*23,32*).

Results

Quantitation of MDR1 RNA

MDR1 RNA was routinely measured by a slot blot procedure in which various amounts of RNA from unknown and known samples were applied to the same blot. A typical RNA slot blot is illustrated in figure 1. RNA from KB-3-1 cells, which are drug sensitive, and RNA from KB-8-5 cells, which are about fivefold multidrug resistant, were included in each blot. Relative to KB-3-1 cells, the KB-8-5 cells have a 30- to 40-fold increase in MDR1 mRNA (*17*). On this basis, the signal intensity of 10 μg of KB-8-5 total RNA was assigned an arbitrary value of 30 U. The value of the signal from each tumor is expressed relative to that of the signal from KB-8-5 RNA. KB-8-5 RNA gives a reproducible and easily detectable signal. To ensure reproducibility of results, we normalized the quantity of RNA loaded for the amount of actin RNA detected. Normalization was usually not necessary, since the amount of RNA was similar in all the blots (fig. 1).

Cancers With High Levels of MDR1 RNA

MDR1 expression was considered to be high if ≥50% of the cancers in each group had detectable levels of MDR1 RNA. In a substantial proportion of the cancers, MDR1 RNA levels were ≥30 U (table 1). Levels of MDR1 RNA were high in several types of untreated cancers, including colon cancer, renal cell carcinoma, hepatoma, adrenocortical carcinoma, pheochromocytoma, islet cell tumor of the pancreas, chronic myelogenous leukemia (CML) in blast crisis, and carcinoid tumor, as well as in cell lines of non-small cell lung cancer with neuroendocrine properties (NSCLC-NE). Typical results from colon and adrenocortical carcinomas are shown in figure 1. The range of signals in four carcinoid tumors is illustrated by the RNA analysis in figure 2.

We performed RNase protection experiments to determine whether MDR1 RNA in these specimens that contained elevated RNA levels initiated only at the downstream promoter used by normal human tissues or also at an upstream promoter detected in some multidrug-resistant cell lines. RNA preparations from most colon carcinomas and adrenocortical cancers and some carcinoid tumors, leukemias, and pheochromocytomas containing ≥30 U of MDR1 RNA were used for these analyses (fig. 3). For these analyses, a 785-bp RNA, representing genomic sequences encompassing the promoter region and >100 bp of the 5' region of the MDR1 mRNA, was hybridized with the RNA samples in solution and then digested with RNase. Two fragments were detected when RNA from KB-8-5 cells and RNA from KB-C1 cells were analyzed, corresponding to two major transcription-initiation sites. The two fragments of 323 and 130 bp, respectively, are indicated by arrows on figure 3 and correspond to mRNA initiated at the upstream and the downstream promoters. In the specimens listed above from patients who had not previously received chemotherapy, only initiation from the downstream site was detected. The amounts of MDR1 RNA detected by RNase protection were similar to those detected by the slot blot analyses, which validates the use of slot blots for detecting MDR1 RNA levels.

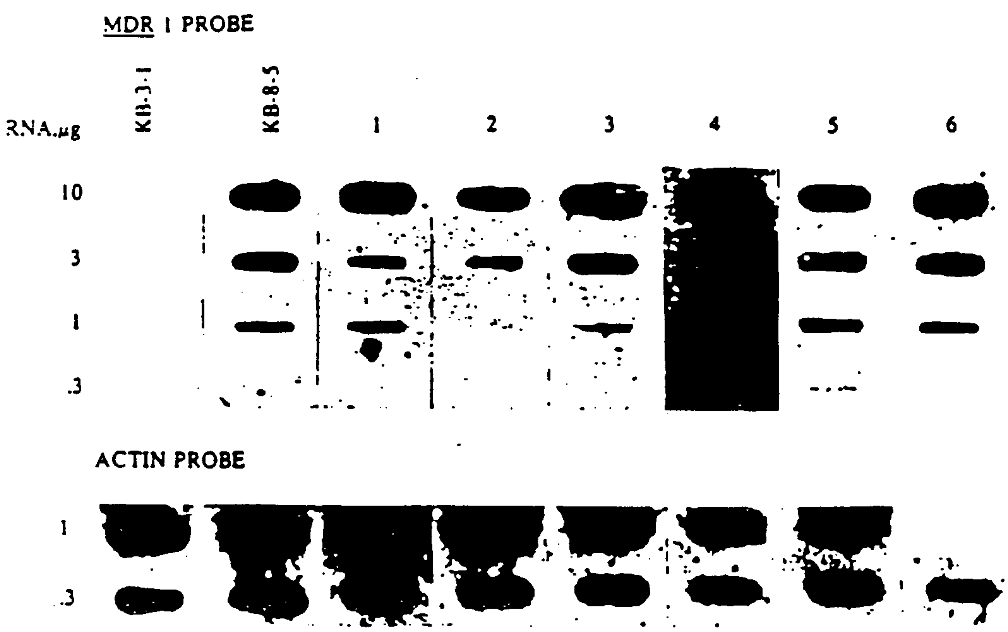

Figure 1. Slot blot analysis of MDR1 expression in untreated human cancers. Lanes 1-3: total RNA samples from colon cancer specimens. Lanes 4-6: RNA samples from adrenocortical carcinomas. Serial dilutions of 10, 3, 1, and 0.3 μg of total RNA were applied to each well. Hybridization of blot with γ-actin probe demonstrated comparable amounts of RNA loaded in all wells. KB-3-1 = drug-sensitive parental KB cell line; KB-8-5 = multidrug-resistant KB subline.

Table 1. Generally high MDR1 RNA levels in untreated cancers*

Cancer type/cell line	Total No. of cancers	No. positive (≥30 U)	No. low positive (2-29 U)	% positive	Reference
Colon carcinoma	41	10	25	85	†,17
Renal cell carcinoma	50	35	5	80	†,19
Hepatoma	12	7	5	100	†
Adrenocortical cancer	9	6	1	77	†,17
Pheochromocytoma	20	11	4	75	†,17
Islet cell tumor of pancreas	4	2	0	50	†
CML (blast crisis)	3	3	0	100	†
Carcinoid tumor	9	2	5	77	†
NSCLC-NE (cell lines)	6	2	3	83	‡

*MDR1 RNA levels were measured by RNA slot blot analysis and are expressed relative to the level in the drug-resistant KB-8-5 cell line, which has been assigned a value of 30 U for the expression of 10 μg of total RNA.
†This work.
‡Lai S-L, Goldstein LJ, Gottesman MM, et al: detailed analysis in preparation.

Cancers With Intermediate Levels of MDR1 RNA

Some untreated cancers were found to have detectable levels of MDR1 RNA ≤50% of the time. Included in this group were adult acute lymphocytic leukemia (ALL), adult ANLL, non-Hodgkin's lymphoma, and neuroblastoma (table 2).

Cancers With Low or Undetectable Levels of MDR1 RNA

A large variety of untreated cancers were found to have generally low (<30 U) or undetectable levels of MDR1 RNA. These cancers included breast cancer, non-small cell lung cancer (NSCLC), bladder cancer, CML in chronic phase, esophageal carcinoma, gastric carcinoma, head and neck cancer, melanoma, mesothelioma, ovarian carcinoma, prostate cancer, sarcoma, small cell lung cancer (SCLC), thymoma, thyroid cancer, and Wilms' tumor (table 3). For nine specimens of squamous cell carcinoma of the lung (included in NSCLC), adjacent normal lung and tumor tissues from each patient were evaluated for expression, and no significant difference in MDR1 RNA expression was found (data not shown).

Figure 4 illustrates the distribution of MDR1 RNA expression in a few representative untreated cancers. Because of the wide range of RNA expression detected, a log scale was used. In this graph it is evident that most of the specimens of adrenocortical cancer and colorectal cancer had relatively high levels of MDR1 RNA, whereas most of the breast cancer specimens and most of the Wilms' tumor specimens had undetectable MDR1 RNA levels, with only a few samples having low MDR1 RNA levels.

Levels of MDR1 RNA in Relapsed Cancers

Cancers that were initially sensitive to chemotherapy but that relapsed after treatment were also examined. Table 4 lists those cancers in which we found high levels of MDR1 RNA after treatment with chemotherapy. These cancers included non-Hodgkin's lymphoma, neuroblastoma, pheochromocytoma, breast cancer, CML in blast crisis, adult ALL, and adult ANLL. In most cases we were not able to obtain specimens from the same patient before and after treatment. However, we did obtain such specimens from one child with ALL (Rothenberg M, Mickley L, Cole D, et al.: manuscript submitted for publication), from one patient with pheochromocytoma, and from two patients with non-Hodgkin's lymphoma. One of the two patients with non-Hodgkin's lymphoma had an MDR1 RNA level of 8 prior to chemotherapy. This patient was then treated with ProMACE-MOPP chemotherapy (cyclophosphamide, doxorubicin, etoposide, prednisone, mechlorethamine, vincristine, and procarbazine). At disease relapse, the MDR1 RNA level increased to 24. This tumor

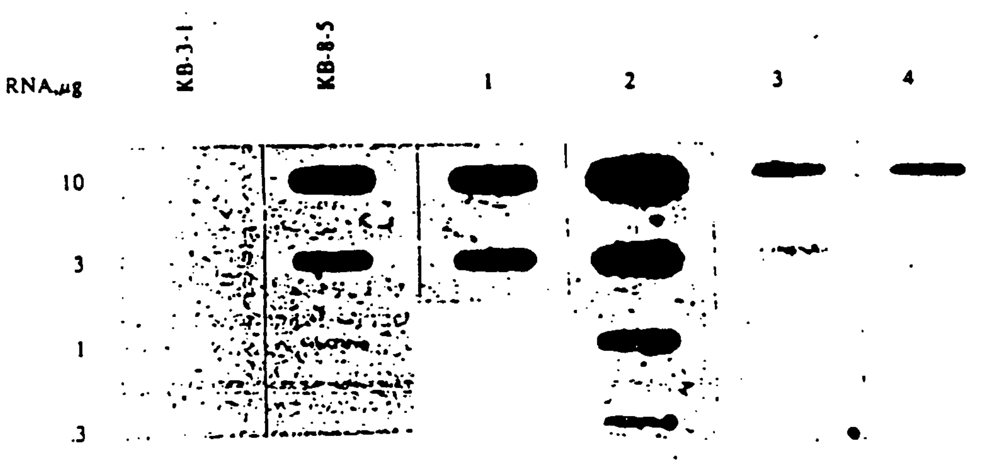

Figure 2. MDR1 expression in carcinoid tumors. Slot blot analysis from four different untreated carcinoid tumors. Serial dilutions of 10, 3, 1, and 0.3 μg of total RNA from each tumor were applied to each well. Hybridization of blot with γ-actin probe demonstrated comparable amounts of RNA loaded in all wells (data not shown). KB-3-1 = drug-sensitive parental KB cell line. KB-8-5 = multidrug-resistant KB subline.

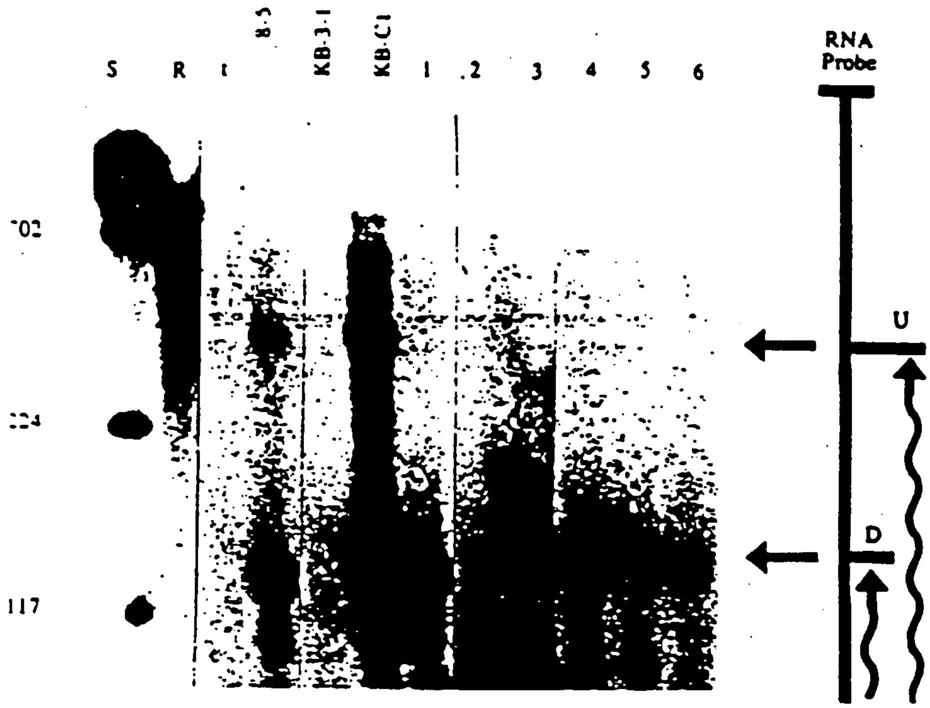

Figure 3. RNase protection assay of untreated cancers with elevated MDR1 RNA levels by slot blot analysis. Samples 1-6 are the same as in fig. 1. In each assay 20 μg of total RNA was used. Two bands were identified when RNA from KB-8-5 cell line and RNA from KB-C1 cell line were used, corresponding to two major initiation sites (designated "U" and "D" for upstream and downstream promoters, respectively). Only the band arising from downstream initiation site is present in these cancers. KB-3-1 = drug-sensitive parental KB cell line; KB-8-5 and KB-C1 = multidrug-resistant KB sublines; S = molecular weight standard; R = riboprobe; t = tRNA.

Table 2. Occasionally high MDR1 RNA levels in untreated cancers

Cancer type	Total No. of cancers	No. positive (≥30 U)	No. low positive (2-29 U)	% positive	Reference
ALL (adult)	15	2	0	13	*
ANLL (adult)	24	3	0	13	*
Non-Hodgkin's lymphoma	18	1	3	22	*
Neuroblastoma	34	1	16	50	†

*This work.
†Goldstein LJ, Fojo A, Gottesman MM, et al: detailed analysis in preparation.

Table 3. Low MDR1 RNA levels in untreated cancers

Cancer type/cell line	Total No. of cancers	No. positive (≥30 U)	No. low positive (2-29 U)	% positive	Reference
Breast cancer	57	0	9	15	*
NSCLC					
Tissue	19	0	7	36	†
Cell lines	30	0	5	16	†
Bladder cancer	6	0	1	16	*
CML (chronic phase)	3	0	0	0	*
Esophageal carcinoma	14	0	0	0	*
Gastric carcinoma	2	0	0	0	*
Head and neck cancer	14	0	0	0	*
Melanoma	3	0	0	0	*
Mesothelioma (cell lines)	20	0	1	5	*
Ovarian carcinoma	16	0	0	0	*
Prostate cancer	3	0	0	0	*
Sarcoma	11	0	0	0	*
SCLC (cell lines)	21‡	0	0	0	†
Thymoma	1	0	0	0	*
Thyroid cancer	4	0	0	0	*
Wilms' tumor	20	0	0	0	*

*This work.
†Lai S-L, Goldstein LJ, Gottesman MM, et al: detailed analysis in preparation.
‡One sample was tumor tissue.

m15

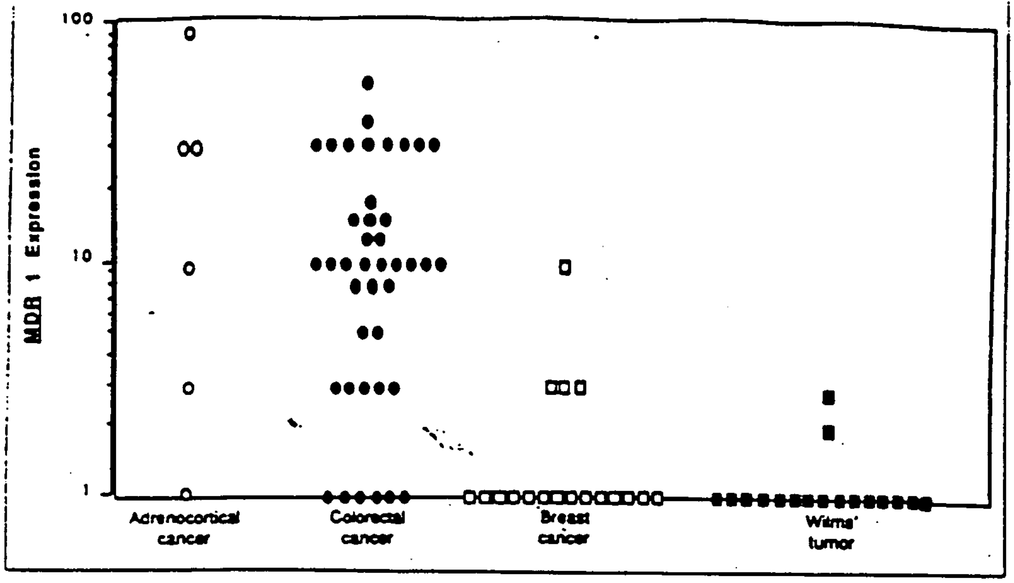

Figure 4. Quantitation of MDR1 expression in representative untreated cancers. Results obtained in slot blot analysis are graphically displayed for adrenocortical cancer, colorectal cancer, breast cancer, and Wilms' tumor. Values of individual tumors were expressed relative to the expression of the multidrug-resistant KB-8-5 cell line, which was arbitrarily assigned a value of 30 U for intensity of 10 μg of total RNA and gave an easily detectable and reproducible signal.

was a nodular, poorly differentiated lymphoma. The other lymphoma specimen studied was also of an indolent histology.

Discussion

Common Expression of MDR1 Gene in Cancers

In this study using RNA slot blot analysis, we have measured the expression of the MDR1 gene in >400 human cancers. Our results show that slot blot analysis is a sensitive method for quantitation of the MDR1 gene expression in human tumors and that many human tumors express MDR1 RNA. We have identified a group of untreated cancers that usually have elevated levels of MDR1 RNA. This group includes colon cancer, adrenocortical cancer, pheochromocytoma, hepatoma, pancreatic carcinoma, and renal cell carcinoma. All of these cancers are derived from tissues that normally have relatively high levels of MDR1 RNA. These findings confirm that the MDR1 gene can continue to be expressed when a normal cell undergoes malignant transformation. All of these cancers are known to be clinically resistant to chemotherapy, and thus the MDR1 gene may be implicated in their intrinsic drug resistance.

Variability in MDR1 Expression

Within the group of cancers with high MDR1 RNA levels, we observed considerable variation from cancer to cancer (fig. 4). For example, the highest MDR1 RNA level in colon cancer was 60 and the lowest was 0; the highest adrenal cancer MDR1 RNA level was 90 and the lowest was 0. This variation was not a technical artifact due to the quality of RNA because all RNA samples were checked for intactness of the RNA by gels and for quantity by analysis of actin RNA levels. However, a number of other factors need further examination. One is the number of cancer cells and stromal cells in each specimen. Stromal cells such as fibroblasts and inflammatory cells tend to have very low MDR1 RNA levels. A second factor is the state of differentiation of the cancer. We have observed in kidney cancers (*19*) and colon cancers (Fojo A: unpublished data) that MDR1 RNA levels tend to be lower in less differentiated cancers. A third factor is the cell type from which the cancer emerges. For example, in the kidney, most cancers showed histological evidence of being derived from proximal tubules (*33*), and the MDR1 gene was preferentially expressed in proximal tubules. In the pancreas, the MDR1 gene was preferentially expressed in collecting ductules. Although we have examined only four pancreatic cancers, the variable expression in this cancer could reflect the origin of the tumor.

Within the various types of lung tumors that have been examined, only one group, NSCLC-NE, tended to have high MDR1 RNA levels. A detailed analysis of this group will be published elsewhere (Lai S-L, Goldstein LJ, Gottesman MM, et al: manuscript in preparation). The group of untreated cancers that occasionally had high MDR1 RNA levels included ALL, ANLL, non-Hodgkin's lymphoma, and neuroblastoma. These cancers are usually sensitive to chemotherapy. It will be important to gather more data to determine if the occasionally elevated levels of MDR1 RNA are associated with the occasional treatment failures seen in these cancers.

Low or undetectable levels of MDR1 RNA were seen in many cancers, including some that are drug sensitive and several others that are generally considered to be resistant or poorly responsive to chemotherapy (e.g., lung cancers). Other mechanisms of drug resistance probably operate in these cancers, or heterogeneity of MDR1 RNA expression could account for resistant subpopulations in these cancers. In the case of breast cancer and NSCLC, some expression of MDR1 RNA was seen in 15%-36% of the tumors examined, which is consistent with the latter possibility. For breast cancer, in particular, in which most of the cells may be stromal,

Table 4. MDR1 RNA levels in tumors relapsing after treatment

Cancer type	Chemotherapy*	Total No. of cancers	No. positive (≥30 U)†	No. low positive (2-29 U)†	% positive	Reference
Non-Hodgkin's lymphoma	−	18	1	3	22	‡
	+	5	1	2	60	
Neuroblastoma	−	34	1	16	50	§
	+	16	5	11	100	
Pheochromocytoma	−	20	11	4	75	‡,17
	+	1	1	0	100	
Breast cancer	−	57	0	9	15	‡
	+	2	0	2	100	
CML						
Chronic phase ¶	−	3	0	0	0	‡
Blast crisis	−	3	3	0	100	
Blast crisis	+	3	2	0	66	**
ALL (adult)	−	15	2	0	13	‡
	+	1	1	0	100	
ANLL (adult)	−	24	3	0	13	‡
	+	5	2	2	80	
ALL (childhood)	−	9††	n	n	11	‡‡
	+	20††	n	n	15	

*− = no chemotherapy; + = chemotherapy given.
†n = not evaluated by quantitative slot blot analysis.
‡This work.
§Goldstein LJ, Fojo A, Gottesman MM, et al: detailed analysis in preparation.
¶Samples from CML in chronic phase and CML in blast crisis with and without chemotherapy are from different patients.
**Pirker R, Goldstein LJ, Ludwig H: detailed analysis in preparation.
††Samples analyzed by Northern blot and RNase protection only.
‡‡Rothenberg M, Mickley L, Cole D, et al: manuscript submitted for publication.

a low level of MDR1 RNA expression could be significant. To investigate the existence of heterogeneous expression, immunohistochemical or in situ hybridization studies of tumor specimens may allow one to distinguish the differential expression of various cell subpopulations.

Acquired Drug Resistance

Several lines of evidence now exist that indicate expression of the MDR1 gene may be partly responsible for acquired clinical drug resistance. In addition to the data reported here showing increased MDR1 RNA levels in ALL, ANLL, lymphoma, breast cancer, pheochromocytoma, CML in blast crisis, and neuroblastoma, antibodies have been used to demonstrate significant levels of P-glycoprotein in some patients with treated ovarian carcinoma, sarcoma, and leukemia (*20-22*). Clearly, further analysis of pretreatment and posttreatment MDR1 RNA levels and/or P-glycoprotein levels in the same patient is needed to prove the association of increased MDR1 RNA levels with acquired drug resistance. In tumors with acquired drug resistance, the measurement of elevated MDR1 RNA levels may help direct further chemotherapy by suggesting that agents affected by the multidrug-resistance phenotype (i.e., *Vinca* alkaloids, anthracyclines, and epipodophyllotoxins) not be used and that alternative treatments be considered.

In addition to observing elevated MDR1 RNA levels in cancers that were intrinsically resistant or that had acquired resistance after treatment, we observed increased MDR1 RNA levels in three patients with CML who had undergone blast crisis. This result raises the possibility that some step that leads to cancer progression, perhaps oncogene activation, could also lead to expression of the MDR1 gene. It has been previously reported that MDR1 RNA levels are elevated in chemically induced tumors of the liver (*34*), a result consistent with simultaneous activation of an oncogene and MDR1 RNA expression.

Characterization of MDR1 RNA in Cancers

RNase protection assays of many cancers that had positive expression confirmed the expression data of slot blot analysis. This protection assay is more specific than the slot blot assay, since the protection assay does not detect RNA transcribed from the closely related MDR2 gene, which has not been associated with multidrug resistance (*12,35*). The RNase protection assay has also allowed us to determine that transcription of the MDR1 gene in cancers of the colon and adrenal gland and carcinoid tumors occurs from the downstream promoter, as does transcription in normal adrenal glands and colon tissues (*23*). Because some drug-resistant tissue culture cell lines also use an upstream promoter, we have continued analyzing cancers to determine which promoters are used. We have found that in the specimens from two of the four children with ALL with elevated MDR1 RNA levels reported here, transcription initiated at both the upstream and downstream promoters; in contrast, in the specimens from the other two children, only the upstream promoter was used (Rothenberg M, Fojo A: unpublished data). The use of two promoter sites has also been seen in both treated and un-

reated adult leukemias and lymphomas that have elevated evels of MDR1 RN/ Goldstein LJ, Pastan I, Gottesman MM: unpublished data. The use of an upstream promoter n drug-resistant tumors suggests a different mechanism of egulation of expression of the MDR1 gene in such instances.

Evidence Linking MDR1 Expression to Multidrug Resistance

Our results have shown that cancers which are clinically drug resistant generally have elevated MDR1 RNA levels. Several lines of evidence suggest that multidrug resistance n cancers with elevated MDR1 expression is due, at least n part, to this expression: (*a*) when full-length cDNAs for the human or mouse MDR1 gene are transfected (*36,37*) or nfected into human cells (*38,39*), these cells become multidrug resistant; (*b*) unselected cell lines from tumors, such as renal adenocarcinoma with elevated MDR1 RNA levels, have a multidrug-resistant phenotype, and their resistance is reversible by use of verapamil and quinidine (*40*), which are inhibitors of the multidrug transporter (*14*); and (*c*) there is some correlation between MDR1 RNA levels in renal adenocarcinomas and resistance of tumor explants to vinblastine (*19*). Based on these results, controlled clinical trials in patients with colorectal and renal cancers are under way with the use of quinidine as a reversing agent in conjunction with cytotoxic therapy including doxorubicin, etoposide, and vinblastine. Another direction of further investigation will be to develop other less toxic reversing agents.

Conclusions

We have measured levels of MDR1 mRNA in many human cancers. We have found elevated expression of the MDR1 gene in certain untreated cancers and in some treated cancers. Although the absence of MDR1 RNA expression in some drug-resistant cancers suggests that other mechanisms of multidrug resistance exist, the widespread occurrence of MDR1 RNA expression in drug-resistant cancers suggests that the MDR1 gene plays an important clinical role in many cancers. We estimate ≈450,000 new cases of cancers expressing the MDR1 gene per year on the basis of our expression data and the incidence of these cancers. Prospective trials correlating measurements of MDR1 RNA expression with clinical response to therapy will determine if MDR1 levels are predictive of response. If they are, MDR1 RNA measurements may be useful in the design or the alteration of chemotherapeutic regimens in individual patients.

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Phase I Trial of Trimetrexate Glucuronate on a Five-Day Bolus Schedule: Clinical Pharmacology and Pharmacodynamics

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Trimetrexate glucuronate (TMTX), a nonclassical folate antagonist, has been evaluated clinically on several schedules. We have studied TMTX administered as an iv bolus for 5 consecutive days every 3 weeks in 35 patients with advanced solid tumors. Drug was given at doses ranging from 7.6 to 18.8 mg/m². The maximal tolerated dose was 13.1 mg/m² per day × 5 for patients without prior myelotoxic treatment and 7.6 mg/m² per day × 5 for previously treated patients. Because of wide individual differences in drug tolerance, dose escalation in 25% increments is recommended for patients not experiencing toxic effects. The dose-limiting toxicity was neutropenia. Rash and mucositis were also significant. TMTX concentrations were measured 1 and 24 hours after each dose, and the data were fit by use of a one-compartment pharmacokinetic model. With this simplified sampling and modeling scheme, the mean total body clearance (± SD) of trimetrexate was 31 ± 20 mL/min per m² and the volume of distribution was 13 ± 7 L/m². Mean plasma concentrations 1 hour after a dose were 1.12, 2.43, 3.33, and 3.25 μmol/L at 7.6, 9.1, 10.9, and 13.1 mg/m², respectively. The mean TMTX concentration (± SD) 24 hours after a dose was 114 ± 87 nmol/L. The mean area under the concentration-versus-time curve at 13.1 mg/m² was 2,266 μmol·min/L. The drug concentration 1 hour after the first dose and the area under the concentration-versus-time curve were highly correlated with leukopenia and thrombocytopenia ($r = .6$ and .65 and $P = .0007$ and .0001, respectively). The maximal tolerated dose on the daily × 5 schedule was 30% of the dose tolerated on an iv bolus schedule. The choice of drug schedule for clinical trials when murine and human pharmacokinetics differ is discussed. Phase II trials are under way with both the iv bolus and the daily × 5 schedules. [J Natl Cancer Inst 1989;81:124–130]

Trimetrexate glucuronate [TMTX; (6-(3,4,5-trimethoxyphenyl)amino methyl)-5-methyl-2,4-quinazoline diamine] is a novel, nonclassical folate antagonist with a broader spectrum of cytotoxicity in preclinical models than methotrexate (*1*). TMTX was also chosen for further clinical studies because it differs from methotrexate in several other pharmacologic properties. TMTX does not enter cells via the reduced folate transport system and is effective in tumor lines exhibiting resistance to methotrexate because of decreased transmembrane transport (*2,3*). A different,

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APPENDIX III

APPENDIX III

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Establishment of Cross-Resistance Profiles for New Agents[1]

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Sublines of murine leukemias (L1210 and P388) and solid tumors selected for resistance to representatives of all of the chemical and functional classes of clinically useful anticancer drugs have been isolated and established in serial in vivo passage and, in some cases, in vitro culture. Extensive resistance, cross-resistance, and collateral-sensitivity patterns have been established with most of the sublines of the drug-resistant murine leukemias under treatment with > 100 different established and clinically useful anticancer drugs or new candidate anticancer drugs currently under study. Patients selected for inclusion in phase I–II trials usually have tumors that have failed to respond to treatment with established clinically useful drugs, either from the start of treatment or during continuing treatment after initial useful response. These treatment failures are no doubt due, in many cases, to drug-resistant tumors if initially unresponsive or to the overgrowth of drug-resistant mutant tumor stem cells in initially responding patients who ultimately failed under continuing treatment. Therefore, the cross-resistance profiles of drug-resistant murine tumors to treatment with new drugs going into phase I–II trials should provide useful guides for patient selection for those trials. Also, these cross-resistance profiles will provide useful information indicating likely biochemical mechanism of action of new drugs with promising anticancer activity, thus guiding drug selection for combination chemotherapy trials in animals or man. Numerous examples of all of the above indications for useful application of such information derived from chemotherapy trials with drug-resistant murine tumors are reported. [Cancer Treat Rep 67:905–922, 1983]

It is commonly observed with drug treatment of both leukemias and solid tumors of man and animals that initially drug-sensitive and responsive tumors become progressively less responsive and ultimately fail to respond during continuing treatment (1,2).

Spontaneous mutation to drug resistance is commonly observed among advanced-stage and grossly evident drug-sensitive murine tumors that are used as experimental models for chemotherapy trials and that were selected to represent the major histologic and organ types of human tumors (2). The rate of spontaneous mutation of murine tumors to resistance to single anticancer drugs varies markedly, being highest to mitotic inhibitors like vincristine (VCR) (1,2); less frequent to antimetabolite drugs like cytarabine (ara-C) (1-3), and lowest to highly active drugs like the alkylating agents, eg, cyclophosphamide (CPA) (1,2). Spontaneous mutation to resistance to all chemical and functional classes of anticancer drugs, including the alkylating agents (4,5), has been observed in mice with total-body burdens of tumor stem cells that are at or below the smallest body burden of all organ or histologic types of cancer in man at a time when the tumor is first clinically detectable (about 10^9 tumor cells in a single focus). Even patients without clinically evident tumor at the start of drug treatment, eg, those receiving drug treatment shortly after surgical removal or radiation kill of evident and accessible primary and/or metastatic lesions, would have had a total-body burden of tumor stem cells prior to surgery and/or radiation large enough to establish a high probability of the presence of residual tumor stem cells which could be resistant to any drug and therefore could be a potential obstacle to curative chemotherapy.

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Since drug-resistant tumor stem cells are an indicated obstacle to curative drug treatment of clinically recognized systemic cancer in man and an objectively established obstacle to curative drug treatment of advanced and grossly evident cancer in experimental animals, it is important to develop methods for control of drug-resistant tumor cells.

We and other investigators are studying the drug-resistant problem in experimental cancer chemotherapy.[3]

When a new anticancer drug is selected for clinical trial on the basis of observed anticancer activity against one or more transplantable animal tumors or human tumor xenografts growing in animals, the patient selection for phase I-II testing is often from previously treated patients who did not respond to treatment or who ultimately relapsed under initially effective and useful drug treatment. If the treatment failure was due to overgrowth of tumor cells resistant to the drugs used in initial treatment, new drugs without activity against tumor cells resistant to the inactive or failing treatment could be prospectively predicted to fail also. Therefore, the sensitivity to the new agent of animal tumor cells selected for resistance to clinically useful drugs that initially or ultimately failed to control each individual patient's tumor should be a prime determinant in patient selection for phase I-II trials. If experimental tumors selected for resistance to the drugs to which the patient's tumor failed to respond were cross-resistant to the new drug entering phase I-II trials, that patient probably would fail under treatment with the new drug in clinical trials, and if a sufficient number of such patients were included in the trials, an otherwise promisingly useful anticancer drug could be overlooked and abandoned.

Studies with animal tumor cells with known drug resistance have provided in the past, and can provide in the future, useful information on all of the basic phenomena relating to drug resistance, biochemical mechanism of action of new drugs, etc. Included in the knowledge to be gained from such studies is an understanding of the mechanism(s) by which originally drug-sensitive tumor stem cells become resistant to drug treatment: eg, enzyme deletion, membrane transport and intracellular retention of drugs, gene amplification resulting in increased formation of the target enzymes, comparison of cytotoxic moieties of alkylating agents, increased DNA repair in drug-resistant tumor stem cells, and increased levels of degradative enzymes, all of which may bear directly and individually or collectively on anticancer activity of the new drug in animals and/or man.

[3]Different techniques and procedures are used by different investigators to collect and interpret data from drug-resistance studies. Since we are only responsible for our own data, we are knowingly, and with apologies to other investigators, limiting this report, with few exceptions, to data that have been collected at Southern Research Institute

Drug-Resistant Murine Leukemias and Solid Tumors Isolated at Southern Research Institute and Available for Study

Drug-resistant sublines of leukemias L1210 and P388 are shown in table 1, and sublines of murine solid tumors that we have isolated under treatment with representatives of all of the currently recognized chemical and functional classes of clinically useful anticancer drugs are shown in table 2. Log_{10} changes in the body burden of the parent drug-sensitive (L1210/0 and P388/0) and the drug-resistant sublines of L1210 and P388 are shown in table 3. In tables 4 and 5, we have tabulated the results of extensive, but still far from complete, chemotherapy trials in which we treated the drug-resistant tumor sublines in the same experiment, in direct "head-to-head" comparison with the parent drug-sensitive tumor, using multiple doses of each drug under study, so that optimal therapeutic response of

TABLE 1.—Murine leukemias selected for resistance to clinically useful anticancer drugs

	Resistant to—*	
	L1210	P388
Alkylating agents		
CPA	X	X
Melphalan	X	X
Carmustine	X	X
Cisplatin	X	X
Antimetabolites		
Ara-C†	X	X
Hydroxyurea	X	
Thiosemicarbazone‡	X	
5-FU		X
5-Azacitidine (Azacyt)		X
6-Thioguanine (6-TG)	X	
6-Mercaptopurine (6-MP)	X	
6-Methylmercaptopurine riboside (6-MeMPR)	X	
Tiazofurin§		X
Ara-A		X
Methotrexate (MTX)		X
DNA binders or intercalators		
Doxorubicin (ADR)		X
Dactinomycin (Act D)		X
Amsacrine (m-AMSA)¶		X
Mitotic inhibitor		
VCR		X
Doubly resistant		
CPA and ara-C	X	
Ara-C and 6-TG	X	
Ara-C and 6-MP	X	
Ara-C and 6-MeMPR	X	
6-MP and 6-MeMPR	X	

* X indicates that drug-resistant tumors have been adapted to growth in cell culture and are currently available.

†We also have an ara-C-resistant mutant of murine acute myelogenous leukemia (AML) (RFM).

‡Pyridine-2-carboxaldehyde thiosemicarbazone.

§2-β-D-Ribofuranosylthiazole-4-carboxamide.

¶Isolated by R. K. Johnson.

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ABLE 2.—Drug-resistant sublines of murine solid tumors isolated at outhern Research Institute during continuing treatment with drugs hat initially caused regression of grossly evident (advanced) tumors

·ug	Tumor
·kylating agents	
·-PAM	M5076 ovarian
Semustine (MeCCNU)	M5076 ovarian
Dacarbazine (DTIC)	Colon 07
DDPt	Colon 04/C
·A binder or intercalator	
ADR	Mammary 16/C
ADR	Mammary 17/A
·ntimetabolites	
Ara-C	Colon 36
·-FU	Colon 12
·riazine antifol (NSC-127755)	Colon 36

.D10 doses, based on change in the body burden of stem cells of both the drug-resistant and the parent g-sensitive tumor lines, could be made. The techni- procedures and the methods of estimating changes .he body burden of tumor stem cells have been de- bed (7) and previously reported (2,8). Simply stated, number of tumor stem cells present at the start at the end of drug treatment are estimated from a plot of the mortality and median lifespan of untreated control mice implanted with $\log_{10}$ dilutions of tumor cells, from 10^7 down to one cell, with both the parent drug-sensitive and the indicated drug-resistant sublines in each experiment. From such plots, one can estimate the number of tumor stem cells present at the start of and at the end of drug treatment, irrespective of size of the tumor implant, the duration of treatment, or other characteristics of the treatment schedule used. In our opinion, this is the most objective and quantitatively precise and reproducible method of estimating therapeutic effectiveness of drug treatment: ie, the change in the body burden of tumor stem cells observed under drug treatment at up to dose-limiting toxicity. Increase in lifespan (ILS), as it is commonly used as an endpoint for estimating therapeutic activity of drug treatment, commonly disregards the duration of treatment and does not provide objective estimates of the body burden of tumor stem cells at the end of drug treatment. If reduction of the body burden of tumor stem cells to below the number capable of re-establishing the ultimately fatal disease is the goal of cancer chemotherapy, as we believe it is, then such estimates of the change in body burden of tumor stem cells by drug treatment (in the absence of cure) are essential for objective evaluation of the therapeutic activity of any drug, drug combination, or treatment protocol.

TABLE 3.—Log_{10} change* in body burden of tumor stem cells after optimal (< LD10) drug treatment

	L1210/0	L1210/drug-resistant	P388/0	P388/drug-resistant
Alkylating agents				
CPA	-6	0	-7	-1
L-PAM	-6	-2	-7	-1
BCNU	-7	-2	-7	-1
DDPt	-5	-1	-6	-2
DNA binders or intercalators				
ADR	-3		-6	-2
Act D	-1		-5	-2
m-AMSA	+1		-6	+3†
Mitotic inhibitor				
VCR	+4		-6	-2
Antimetabolites				
Ara-C	-6	+1	-6	-1
Hydroxyurea	-4	+3		
Thiosemicarbazone	-8	+3		
5-FU	+1		-5	+2
Azacyt	-5		-6	+3
6-TG	-3	0	-3	
6-MP	-2	+1	0	
6-MeMPR	-4	-1	-1	
Tiazofurin	-3		-3	+2
Ara-A	-4		-6	+2
MTX	0		-3	+3

*Log_{10} change = net $\log_{10}$ change in tumor stem cell population at the end of treatment as compared to the start of treatment: eg, a -3 log change means that there was a 99.9% reduction and a +3 log change means that there was a 1000-fold increase in tumor burden at the end of treatment.

†Data of Johnson and Howard (ref 6).

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TABLE 4.—Log10 change* in the body burden of parent drug-sensitive and selected drug-resistant leukemia L1210 stem cell populations by drug treatment (at ≤ LD10 dose from dose-response studies) of BDF1 or CDF1 mice implanted ip with 10^5-10^6 tumor stem cells and treated ip as indicated

Agent	NSC No.	Treatment schedule†	L1210 Parent/0	/CPA	/BCNU	/L-PAM	/DDPt	/Ara-C	/6-TG	/6-MP
Alkylating agents										
Mechlorethamine	762	A	-3			3				
CPA	26271	A	-6	0	-6	4	-6	-6		
Ifosfamide (IFA)	109724	A	-7	-2						
4-Hydroperoxyisophosphamide	227114	A	-7	-2‡						
4-Hydroxyisophosphamide	196641	A	5	-2‡						
4-Peroxycyclophosphamide	179686	A	-7	-1‡						
4-Hydroxycyclophosphamide	196562	A	-4	0‡						
Phosphoramide mustard (PM)	69945	A	-6	-5		4				
Isophosphoramide mustard (IPM)	297900	A	-6	-6‡						
L-PAM	8806	A	-6	-5	-6	2	-4			
Peptichemio	247516	A	-4‡	-3‡	-2‡	0‡				
Asaley	167780	A	-2			0				
Chlorambucil	3088	D	+2‡	+2‡						
Uracil mustard	34462	A	-3	-2						
Piperazinedione	135758	A	-5	-4	-4	5	-5			
		D	-2	+2‡	0	5		0‡		
Thiotepa	6396	A	-5	-2	-2	2	-3			
Mitomycin	26980	A	-4	-5‡		3				
Dianhydrogalactitol	132313	A	-5	-5	-3	4	-4			
		B	-5			2				
BCNU	409962	A	-7	-7	-2	-6	-7	-6		
Lomustine	79037	A	-7	-6	-2	7	-7			
MeCCNU	95441	A	-7	-7	-3	7	-7			
PCNU	95466	A	-7			7				
Streptozocin	85998	A	0	-1	-1	0				
Chlorozotocin	178248	A	-7	-6	-4	-6	-6			
BIC	82196	A	-7	-6	0	-6	-6			
DTIC	45388	A	-2‡		-1‡					
		D	+1			+2				
Platinum										
DDPt	119875	A	-5	-3‡	-5		-1			
		B	-3				+3			
		C	-5	-5			+3			
		D	+2				+3			
155	194814	A	-5‡				-5‡			
(Ethylenediamine)(malonato)	146068	D	+2‡				+3‡			
Cyclobutane	241240	D	+2‡				+3‡			
Diisopropylamine	256927	C	-1‡	-2‡	+1‡	+2‡				
		D	+1‡				+2‡			
Dicyclopentylamine	178858	C	-3‡	+1‡	+2‡	+3‡				
Carboxyphthalato-1,2-diaminocyclohexane	271674	A	-6			-4	-4‡			
		B	-6				-7			
		D	-6			-7	-7			
Dichloro(2, 2-dimethyl-1,3-propanediamine-N, N'), (SP-4-2)-	349946	B	+2‡				+3‡			

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Procarbazine	77213	D	+1	+2	+2	+3			
DNA binders or intercalators									
ADR	123127	A	-3			-1			
Act D	3053	A	-1			-2			
Antimetabolites									
MTX	740	C	-2						-6
		D	0	0‡	+2‡	+3	+1‡	-3	-4
Dichloromethotrexate	29630	D	-1						-5‡
10-Deaza-aminopterin	311469	D	+2‡						-4‡
5-Methyltetrahydrohomofolic acid (5-MeTHHF)	139490	D	+3						+2
Quinazoline antifolate	327182	C	+1						+1
2,4-Quinazolinediamine, 6-[[(3,4,5-trimethoxyphenyl) amino]methyl]-, 2-hydroxy ethanesulfonate (1:1)	328564	D	+2‡						-2‡
L-Glutamic acid, N-[4-[[(2,4-diamino-6-pyrido[2,3-d]pyrimidinyl)methyl]methylamino] benzoyl]-, dihydrate	344280	C	-1‡						-4‡
L-Glutamic acid, N-[4-[[(2,4-diamino-6-pyrido[2,3-d] pyrimidinyl)methyl]amino benzoyl]-, diethyl ester	346890	C	-1‡						-6‡
Baker's antifol	139105	D	+4						+3‡
Triazine antifol	127755	D	+3						+3
DDMP	19494	D	+3						+3
6-MP	755	A	-2				-2	-1‡	+1
		D	+3	+3	+3			+3	+4
6-MeMPR	40774	A	-4					-3‡	-1‡
		D	+1					+2‡	+2‡
6-TG	752	A	-3				-3	0	
		D	+3	+3		+4			
Ara-A + 2'-deoxycoformycin (2'dCF)	404241 218321	E	-4				-1		-5
2-Fluoro-ara-A	118218	D	-5				+4		
2-Fluoro-ara-AMP (sodium)	328002	A	-3‡				-3‡		
		D	0‡				+2		
5'-Formyl-2-F-ara-A	302067	D	-2				+4		
Ara-C	63878	E	-6		-6		+4		
PalmO-ara-C	135962	A	-6	-6	-5	6	+1		
		D	-5						-8
5-FU	19893	D	+1	+1‡	+2‡	+1	+2		+2
Floxuridine (5-FUdR)	27640	D	+3	+3‡	+3‡				
6-Azauridine	32074	D	+3				+4		
3-Deazauridine	126849	D	+2				-6		
		E	+2				-6		
5'-O-Acetyl-3-deazauridine	329943	D	+2				-6		
5'-O-Propionyl-3-deazauridine	329944	D	+1				-6		
5'-O-N-Butyryl-3-deazauridine	329945	D	+2				-6		
5'-O-Pivalyl-3-deazauridine	329946	D	+2				-6		

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TABLE 4.—*Continued*

Agent	NSC No.	Treatment schedule†	L1210 Parent/0	/CPA	/BCNU	/L-PAM	/DDpt	/Ara-C	/6-TG	/6-MP
5'-O-Benzoyl-3-deazauridine	328947	D	+2					-6		
2',3',4'-Tri-O-acetyl-3-deazauridine	328948	D	+2					-6		
4'-O-(Adamantan-1-carbonyl)-3-deazauridine	328949	D	+2					-6		
5'-O-Palmitoyl-3-deazauridine	340570	D	+2					-6		
5-Azacitidine	102816	D	-5			-5		-5‡		
Dihydro-5-azacitidine	264880	D	-2‡					-5‡		
PALA	224131	D	+4					+4		+3
Pyrazofurin	143095	D	+1‡					+3‡		
Hydroxyurea	32065	E	-4§					-4§		
Guanazole	1895	E	-4§					-4§		
P-2-TSC	729	E	-5					-1		
Mitotic inhibitor										
VCR	67574	B	+4			+4				

*Log_{10} change = net log_{10} change in tumor stem cell population at the end of treatment as compared to the start of treatment; eg, a -3 log change means that there was a 99.9% reduction and a +3 log change means there was a 1000-fold increase in tumor burden at the end of treatment.

†A = single-dose; B = q4d × 3; C = qd 1-5 days; D = qd 1-9 days; and E = q3h × 8 and q4d × 3.

‡Single experiment.

§10^6 tumor stem cell implant.

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sistance, Cross-Resistance, and Collateral nsitivity of Selected Drug-Resistant L1210 and 38 Leukemias to Clinically Useful Anticancer ;gs and to New Drugs Under Development

he data shown in tables 4 and 5 were usually ob- ed by treatment with the optimal dose (from a -response study) and the optimal treatment sched- for the parent drug-sensitive tumor in each case. compounds listed in tables 4 and 5 are representa- of the clinically useful drugs in each of the chem- and functional classes of anticancer drugs, as well number of other compounds that are now or have under investigation as potentially useful new s.

nerally consistent observations among the drug-re- it lines of L1210 and P388 treated with clinically l anticancer drugs or drugs in development as n in tables 4 and 5 are as follows.

Except for cross-resistance to other drugs with ir chemical structure and/or biologic function, tion of tumor cells to resistance to one drug usual- s not result in resistance to other drugs, particu- those of other functional classes. For example, cells selected for resistance to antimetabolite usually retain full sensitivity to alkylating , to drugs that bind to or intercalate with DNA, mitotic inhibitors such as VCR.

umor cells resistant to an anticancer drug are cross-resistant to structural congeners of that eg, 6-TG versus L1210/6-MP and congeners of ersus L1210/CPA. However, exceptions to this principle are common. (*a*) L1210/CPA is cross- nt to a number of analogs of CPA but retains nsitivity in vivo to PM and IPM. The lack of sistance of L1210/CPA to PM and IPM may be increased production of aldehyde dehydrogenase 10/CPA (9,10), possibly due to gene amplifica- heoretically, adequate levels of PM and IPM be formed by normal metabolism of CPA or IFA refore, resistance to CPA would not be evident. of CPA or IFA (at doses up to dose-limiting tox- control CPA-resistant tumor cells is not clearly od but may be due to different pharmaco- ircumstances (rate of production of PM from IPM from IFA), rate of cellular uptake and/or ion, or other unrecognized variables. These ies are supported by the fact that CPA is cytotoxic in vivo for L1210/CPA at single about 5 times the LD10.[4] (*b*) L1210/DDPt is stant to most analogs of DDPt but remains sitive to the carboxyphthalato analog (NSC-271674) (table 4). The significance of these observations is not clear since both P388/0 and P388/DDPt are essentially insensitive to NSC-271674 (table 5). (*c*) L1210/ara-C and P388/ara-C are sensitive to ara-A + 2'dCF, a potent deaminase inhibitor, but are cross-resistant to 2-fluoro-ara-A on an every day, Day 1-9 treatment schedule, while 2-fluoro-ara-A is active against P388/ara-A (table 5). These activities are to be expected since it is known that 2-fluoro-ara-A is phosphorylated by CdR kinase and not by AdR kinase. Therefore, these failures to predict cross-resistance based on similar chemical structure between ara-A and 2-fluoro-ara-A are well-understood on a biochemical basis (3,8,11).

Collateral Sensitivity

Numerous examples of collateral sensitivity (CS)[5] of L1210 and P388, selected for resistance to antimetabolite drugs, are evident in the data presented in tables 4 and 5. These examples are listed in table 6 for purposes of discussion. The first marked CS reported was that of 6-MP-resistant L1210 cells (L1210/6-MP) to treatment with MTX (13). A similar CS of human leukemia cells may contribute to the clinical effectiveness of VAMP (VCR, MTX, 6-MP, and prednisone) used in treating acute lymphatic leukemia (ALL) of children, although such CS of 6-MP-resistant ALL cells to MTX in man has never been objectively investigated and established. However, CS may be an asset worth investigating and attempting to exploit in relation to the control of ara-C-resistant tumor cells in man. With both L1210/ara-C and P388/ara-C, a number of remarkable examples of CS to other antimetabolite drugs have been observed (table 6).

The quantitatively greatest CS shown in table 6 is that of L1210/ara-C to 3-deazauridine (about 8 orders of magnitude greater cell kill of L1210/ara-C than of L1210/0 at equitoxic doses; ie, at < LD10 doses). 3-Deazauridine has been tried, without therapeutic response, in patients with AML who had had extensive prior treatment with ara-C and were in relapse, possibly due to overgrowth of ara-C-resistant AML cells. We have tried ara-C plus 3-deazauridine against body burdens of L1210/0 cells large enough to predict treatment failure due to the overgrowth of L1210/ara-C. No therapeutic gain over that from ara-C alone was seen. These clinical and laboratory failures to demonstrate therapeutic improvement associated with CS of ara-C-resistant cells may be due to the greater-than-additive toxicity for vital normal cells when 3-deazauridine and ara-C are used together (*see* ref 3 for a discussion of these clinical and laboratory studies).

L1210/6-MP shows CS to MTX and to some, but not all, new compounds that have been synthesized in drug development programs seeking new and improved

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hed data from Southern Research Institute.

l sensitivity has been defined as increased sensitivity of a nt line of tumor cells to another drug over that seen in the sensitive cells (12)

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TABLE 5.—Log₁₀ change* in the body burden of parent drug-sensitive and selected drug-resistant leukemia P388 stem cell populations by drug treatment (at ≤ LD10 dose from dose-response studies) of BDF1 or CDF1 mice implanted ip with 10^6-10^7 tumor stem cells and treated ip as indicated

Agent	NSC No.	Treatment schedule†	P388 Parent/0	/VCR	/ADR	/Act D	/CPA	/L-PAM	/BCNU	/DDPt	/Ara-C	/Ara-A	/5-FU	/MTX	/Asacyt
Alkylating agents															
CPA	26271	A	-7	-7	-7	-7	-1	-7	-6		-6				
IFA	109724	A	-7‡				-4‡								
4-Hydroperoxyisophosphamide	227114	A	-7‡				-6‡								
PM	69945	A	-7				-7	-6							
IPM	297900	A	-6				-6								
L-PAM	8806	A	-7	-7	-7	-7	-7	-1	-6‡	-7‡					
Peptichemio	247516	A	-6				-6‡	-2							
Chlorambucil	3088	D	+1‡				+1‡								
Mitolactol	104800	D	+1‡		+1‡										
Piperazinedione	135758	A	-6	-6	-6	-5	-6	-3	-6‡						
Mitomycin	26980	A	-6				-4	-2	-6						
Dianhydrogalactitol	132313	A	-6				-6	-3	-6						
		B	-6					-1							
BCNU	409962	A	-7	-7	-7	-7	-7	-7	-1						
PCNU	95466	A	-7					-7‡							
ACNU	245382	A	-7					-7‡							
BIC	82196	A	-6				-6	-6‡							
Platinum															
DDPt	119875	A	-6					-2		-2					
		B	-6					+2	-6	+3					
		C	-6	-4	-6	-6	-6			+3					
Pt-155	194814	B	-1‡							0‡					
Carboxyphthalato-1,2-diaminocyclohexane	271674	B	+1‡							+2‡					
Indicine-N-oxide	132319	D	+3				0								
Procarbazine	77213	D	+1	+3	+3	+1	+3								
DNA binders or intercalators															
Act D	3053	A	-5	-5	-1	-2	-6	[illegible]							
Azetomycin I	244392	B	-0‡			+1‡									
Azetomycin II	244393	B	-6‡			+1‡									
ADR	123127	A	[illegible]	-6	-2	-5	-6	[illegible]			-6			-6‡	
Daunorubicin	82151	A	-6	0	-1‡										
Zorubicin	164011	A	-6‡		0‡										
AD-32	246131	C	-6	-6											
Aclarubicin	208734	B	0‡		+3‡										
Carminomycin	180024	B	+2‡		+3‡										
Marcellomycin	265211	B	+2‡		+3‡										
Nogamycin	265450	B	+2‡		+3‡		-7				-7				
Anthracenedione	196473	D	-6		+2										
Anthracenedione, diacetate	287513	D	-6		+2		-6								
Anthracenedione, dihydroxyl	279836	A	-7	-6	-1	-6									
		D	-1		+1										
m-AMSA	249992	B	-6	+2‡	+2	-2‡									

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MTX	740	C	-3		[illegible]	[illegible]				+1						
		D	-3	-4					-2‡	+3						
Quinazoline antifol	327182	C	0							+1						
2,4-Quinazolinediamine, 5-methyl-6-[[(3,4,5-trimethoxyphenyl)amino]methyl]-, 2-hydroxyethanesulfonate (1:1)	328564	D	-4‡							0‡						
Baker's antifol	139105	D	+2						+2‡							
Triazine antifol	127755	D	-2							0						
10-Deaza-aminopterin	311469	D	-2‡					+2‡	+2							
DDMP	19494	D	+2‡	+2‡												
6-MP	755	A	0													
		D	+2	+2	+2	+2										
6-MeMPR	40774	A	-1													
6-TG	752	A	-3													
		D	+2	+2‡	+2‡	+2‡							-3			
Ara-A +	404241+	D	-6												+2‡	
2'dCF	218321	E	-6										0			
L-Alanosine, sodium salt	153353	D	+2										-3		-2	
Tiazofurin	286193	D	-3									-6			+2	
2-Fluoro-ara-AMP (sodium)	328002	A	-3										+2			
		D	-4										+2			
2-Fluoro-ara-AMP (ammonium)	311463	D	-2									-1				
Ara-C	63878	D	-1									-6				
PalmO-ara-C	135962	A	-3	-6	-6	-6						-2‡	-3‡			-3‡
Ara-CDP-diglyceride	—	C	-6									-3‡	-4‡			
5-FU	19893	A	-3		-3								+1‡			
		D	-5	-3	-6	-3						+3				
5-FUdR	27640	A	-3‡				-6	-8				-1	-8	-7	-6‡	
		D	0	-3	-1‡	-2						+2				
3-Deazauridine	126849	D	+3				-1					-6	-5			
5-Azacitidine	102816	A	-3				-1					-8	+1‡	+2		-1
		D	-8									-3‡				+3
Dihydro-5-azacitidine	264880	D	-1													+3
Ara-5-azacytosine	281272	D	-5‡									-1		+3		0‡
PALA	224131	D	+2	+2‡	+2‡								-3‡			
Pyrazofurin	143095	D	+3									-8				
Acivicin	163501	D	-1									-8				
							+2					-3		0		
Protein synthesis inhibitors												-2		+2		
Homoharringtonine	141633	D	-2	+1	+2							-4				
Bruceantin	165563	D	-2		+1‡							-6				
Mitotic inhibitors							-2‡									
VCR	67574	A	-6	-2												
		B	-6	+2	+3	+3										
Vinblastine	49842	B	-3	0			-6	0			-4	-4‡			-4	
Vindesine	245467	B	-5	+2												
Bis(N-ethylidene vindesine) disulfide, disulfate	277096	B	-4	-1							-6‡					
Etoposide	141540	B	-7	-8	+1	-5‡										
Maytansine	153858	E	-6‡	-2‡			-7	-7								

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TABLE 5.—*continued*

Agent	NSC No.	Treatment schedule†	P388												
			Parent/0	/VCR	/ADR	/Act D	/CPA	/L-PAM	/BCNU	/DDpt	/Ara-C	/Ara-A	/5-FU	/MTX	/Azacyt
Carbamic acid, 6-[illegible] dihydro-3-[[met[illegible] amino]methyl[illegible] pyrazine-7-yl, [illegible]	181928	A	-5	-5‡										-2‡	
Carbamic acid, 6-[illegible] dihydro-3-pheny[illegible] [3,4-b]pyrazine-7-yl-, ethyl ester	330770	A	-4	-3											
Carbamic acid, 5-amino-1,2-dihydro-3-[[(4-methoxy-phenyl) methylamino]methyl]-pyrido[3,4-b]pyrazine-, ethyl ester	269416	A	-5‡											-6‡	

*Log_{10} change = net log_{10} change in tumor stem cell population at the end of treatment as compared to the start of treatment; eg, a -3 log change means that there was a 99.9% reduction and a +3 log change means that there was a 1000-fold increase in tumor burden at the end of treatment.

†Schedule: A = single-dose; B = q4d × 3; C = qd 1-5 days; D = qd 1-9 days; and E = q3h × 8 and q4d × 3.

‡Single experiment.

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Table 6 —

Sensitivity, Cross-Resistance, and Collateral Sensitivity of Antimetabolite-Resistant Leukemia L1210 and P388

Agent	NSC No.	Log10 Change[a] Change in Tumor Stem Cells at End of Optimal Therapy					
		10^5 Stem Cell Implant			10^6 Stem Cell Implant		
		L1210/0	L1210/ARA-C	L1210/6-MP	P388/0	P388/ARA-C	P388/ARA-A
Ara-C (Palmitate)	135962	-6	+1	-6	-6	-1	-6
Hydroxyurea	32065	-4*	-4*				
Guanazole	1895	-4*	-4*				
P-2-TSC	729	-5	-1				
Ara-A + 2'dCF	404241 + 218321	-4**	-1**	-5	-6	-6	+2
2-F-Ara-A	118218	-5**	+4**				
2-F-Ara-AMP	328002				-4**	+3**	-4**
Tiazofurin	286193	-1**		-1**	-3	-6 ***	
L-Alanosine	153353				+2	-1	
3-Deazauridine	126849	+2	-6		+3	-1	
Dihydro-5-azacytidine	264880	-2**	-5**		-1	-6	
5-FU	19893	+1	+2		-3	-6	
PALA	224131	+4	+4		+2	-3	
Pyrazofurin	143095				+3	-2	
Acivicin	163501				-1	-4	
Homoharringtonine	141633				-2	-6	
MTX	740	0		-4			
Dichloro-MTX	29630	-1		-5**			
3-Deaza-MTX	344280	-1**		-4**			
5-Deaza-aminopterin, Diethyl Ester	346890	-1**		-6**			
10-Deaza-aminopterin	311469	+2**		-4**			
Trimetrexate	328564	+2**		-2**			
5-Methyltetrahydrohomofolic Acid	139490	+3		+2			
Quinazoline Antifol	327182	+1		+1			
Baker's Antifol	139105	+4		+3**			
Triazine Antifol	127755	+3		+3			
DDMP	19494	+3		+3			
CPA	26271	-6	-6		-6	-6	

*10^4 Cell implant.

**Single experiment.

***Examples of collateral sensitivity are shown in the enclosed boxes.

[a]Log10 change = Net log10 change in tumor stem cell population at the end of Rx as compared to the start of Rx; e g , a -3 log change means that there was a 99.9% reduction and a +3 log change means that there was a 1000-fold increase in tumor burden at the end of Rx.

ITX-like drugs. Data in [illegible] indicate that CS of .1210/6-MP separates these [illegible] drugs into two obiously different groups, based on both activity against .1210/0 and CS of L1210/6-MP. Perhaps this suggests nat new drugs which bind tightly to dihydrofolate reuctase (DHFR) or inhibit thymidylate synthetase hould be tested against L1210/0 and L1210/6-MP for omparative MTX-like activity and also against one or ore of a spectrum of solid tumors, eg, colon adenorcinomas 10, 12, 36, and/or 38 as well as ovarian M5076, all of which are markedly responsive to the new triazine antifol NSC-127755 (14) and more sensitive to Baker's antifol (NSC-139105), DDMP (NSC-19494), and/or 5-MeTHHF (NSC-139490) than to MTX.[e]

The broad-based CS of P388/ara-C to a number of inhibitors of de novo purine or pyrimidine synthesis is also shown in table 6. These drugs might be considered for use in treating patients bearing tumors that initial-

[e]Unpublished data from Southern Research Institute.

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TABLE 7.—In vivo tumor cell kill with PALA (optimum response, treatment every day, Days 1-9, at ≤ LD10 doses*)

Tumor	Implant size, ip	Tumor stem cells present at end of treatment	Log_{10} change† in tumor cell population under treatment with PALA
P388/0	10^6	2×10^8	+2
	10^6	9×10^8	+3
	10^6	9×10^8	+3
P388/ara-C	10^6	3×10^2	-4
	10^6	2×10^4	-2
	10^6	5×10^3	-2
	10^6	1×10^3	-3
	10^6	1×10^4	-2
	10^6	2×10^3	-3
	10^6	2×10^4	-2
	10^6	5×10^3	-3

*The LD_{10} dose of PALA, ip, daily, Days 1-9, is approximately 200 mg/kg/dose.

†Log_{10} change = net log_{10} change in tumor stem cell population at the end of treatment as compared to the start of drug treatment; eg, a -3 log change means that there was a 99.9% reduction and a +3 log change means that there was a 1000-fold increase in tumor burden at the end of treatment.

ly responded to ara-C but are failing during continuing treatment, presumably due to overgrowth of ara-C-resistant cells, or for use in combination with ara-C to attempt to control the ara-C-resistant tumor cells as they appear.

Data in table 6 indicate that the body burden of L1210/0 stem cells increases by about 2 orders of magnitude under treatment up to dose-limiting toxicity with PALA, but the body burden of P388/ara-C stem cells is reduced by about 3 orders of magnitude under the same treatment with PALA. Data in table 7 show the consistent reproducibility of the CS of P388/ara-C to PALA. It should be pointed out and always remembered that such consistent and reproducible biologic responses can be accomplished only by diligent control of all variables (tumor, host mice, drug preparation, and most importantly, proper data evaluation). We have tested ara-C plus PALA against body burdens of P388/0 stem cells in excess (about 10^9 P388/0 stem cells) of the curative potential (because of overgrowth of P388/ara-C) of ara-C when used alone. Marked therapeutic synergism, probably due to control of P388/ara-C that is CS to PALA, was repeatedly observed (3), thus establishing the validity of the thesis that CS to other drugs can be therapeutically exploited, at least in murine tumor systems, if drug treatment failure may be due to overgrowth of drug-resistant tumor cells that are CS to the second drug.

There are two other important points based on the data in table 6 that deserve serious consideration:

1. Usually drugs that are inactive (do not reduce the body burden of tumor stem cells when used alone) are not considered for inclusion in drug treatment protocols. PALA, pyrazofurin, L-alanosine, and perhaps acivicin might not be considered for clinical trial because the body burden of stem cells of P388/0, generally considered to be highly sensitive to most clinically useful anticancer drugs, increases markedly under treatment up to dose-limiting toxicity with all of these drugs except acivicin. Clearly, their marked cytotoxic activity against ara-C-resistant tumor stem cells should make them prime candidates for inclusion in drug combinations being considered for clinical trial if ara-C is included in the drug combination and large body burdens of tumor stem cells (likely to contain ara-C-resistant cells) are present at start of treatment.

2. Since very sensitive, but consistent and reproducible, tumor systems are needed for detection of candidate antitumor drugs, particularly in screening for new drugs, serious consideration should be given to substituting P388/ara-C for P388/0 as the primary screen, or as one of the primary screens, if several are used. If net reduction of the body burden of tumor stem cells is the requirement for antitumor activity (as it should be), the P388/ara-C would easily detect L-alanosine, 3-deazauridine, PALA, pyrazofurin, and perhaps numerous other drugs that are now either discarded by the screen or considered to be of marginal interest because of relative insensitivity of the tumors, including P388, currently being used to screen for new agents.

Pleiotropic Phenotypic Drug Resistance

Variable cross-resistance patterns are seen among tumor cells selected for resistance to large polycyclic anticancer drugs that have markedly different biologic inhibitory activities and chemical structures, among them some drugs that bind to or intercalate with DNA, some inhibitors of mitosis, and others that inhibit protein synthesis. The concept that resistance to multiple drugs may

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result from a single pleiotropic mutation to multiple drug resistance, with all of its negative implications to controlling overgrowth of drug-resistant cells in large body burdens of tumor stem cells by combination chemotherapy, given either simultaneously or sequentially, has been reported by Ling (15,16) and included in these proceedings by Ling, Biedler, and others.

Appearance of tumor cells that are resistant to a number of anticancer drugs with markedly different chemical structures and likely different biologic mechanisms of action during in vivo treatment with a single drug has been repeatedly seen by us (2), and others (6,17,18). The drugs involved are primarily large polycyclic compounds known or presumed to bind to or intercalate with DNA, to bind to tubulin or otherwise inhibit mitosis, or to inhibit protein synthesis. However, the variability of the cross-resistance patterns among these drugs is such that no prospective prediction that resistance to one of these drugs confers resistance to others among this group of compounds can be made with great confidence of accuracy, in the absence of objective data.

Murine Leukemias

Table 8 shows some of the cross-resistances that we have seen in therapy trials with P388 cells selected for resistance to some of these large polycyclic anticancer drugs. While cross-resistance among these drugs is common, obvious exceptions are also common; eg, P388/ADR shows the greatest consistency of cross-resistance to other large polycyclic drugs, but the P388/Act D retains essentially full sensitivity to ADR, dihydroxyanthracenedione (mitoxantrone), and VP-16-213. P388/VCR retains marked sensitivity to ADR, dihydroxyanthracenedione, Act D, and VP-16-213; however, P388/VCR and P388/ADR both are markedly cross-resistant to homoharringtonine, while P388/ara-C shows marked CS to homoharringtonine (19) (table 6).

Mammary Adenocarcinoma 16/C

We have recently observed resistance and cross-resistance to members of this group of large polycyclic anticancer drugs with at least one drug-sensitive solid tumor, suggesting that spontaneous pleiotropic mutation to multiple drug resistance (ADR and VCR) also may occur in advanced solid tumors. These data are shown in figures 1-3. $B6C3F_1$ (C57BL/6 ♀ × C3H ♂) mice bearing sc implanted mammary adenocarcinoma 16/C ranging in size from 50 to 500 mg (mean size, about 280 mg) were treated with ADR alone or CPA + ADR + 5-FU (CAF). Optimal regression responses of individual tumors are shown in figure 1. Overgrowth of presumed ADR-resistant tumors occurred early during treatment with ADR alone (fig 1, panel 2) and later with CAF (fig 1, panel 3). Tumor 8 (fig 1, panel 3), 288 mg at start of CAF treatment, regressed to below palpable size by Day 21 (11 days after start of CAF treatment and 4 days after second treatment) and grossly evident tumor reappeared on Day 38 after implant and 7 days before last treatment. On Day 71 after implant (26 days after last treatment), tumor-bearing Mouse 8 was killed and the tumor was passed to

TABLE 8.—Sensitivity, resistance, and cross-resistance of P388/0 leukemia and sublines selected for resistance to ADR, Act D, VCR, or *m*-AMSA and of a colchicine-resistant subline of Chinese hamster ovary (CHO) cells to treatment with a variety of polycyclic anticancer drugs (DNA binders, mitotic inhibitors, and inhibitors of protein synthesis)

Drug	NSC No.	Log_{10} change* in body burden of tumor stem cells after optimal (≤ LD10) drug treatment: P388/0	P388/ADR	P388/Act D	P388/VCR	CHO cells: CH^RC5/colchicine†
DNA binders or intercalators						
ADR	23127	-6	-2	-5	-5	
Daunorubicin	82151	-6	-1‡		-0	CR
Anthracenedione	279836	-7	-1	-6	-6	CR
m-AMSA	249992	-6	+2	-2‡	+2‡	
Act D	3053	-5	-1	-2	-5	
Mitotic inhibitors						
VCR	67574	-6	+3	+3	+2	
Vinblastine	49842	-3			0	
VP-16-213	141540	-7	+1	-5‡	-6	CR
Maytansine	153858	-6‡	-2‡			
Colchicine	757					R
Protein inhibitors						
Homoharringtonine	141633	-2	+2		+1	
Bruceantin	165563	-2	+1‡			

*Log_{10} change = net log_{10} change in tumor stem cell population at the end of treatment as compared to the start of treatment; eg, a -3 log change means that there was a 99.9% reduction and a +3 log change means that there was a 1000-fold increase in tumor burden at the end of treatment.
†In vitro: CR = cross-resistant and R = resistant (ref 16).
‡Single experiment.

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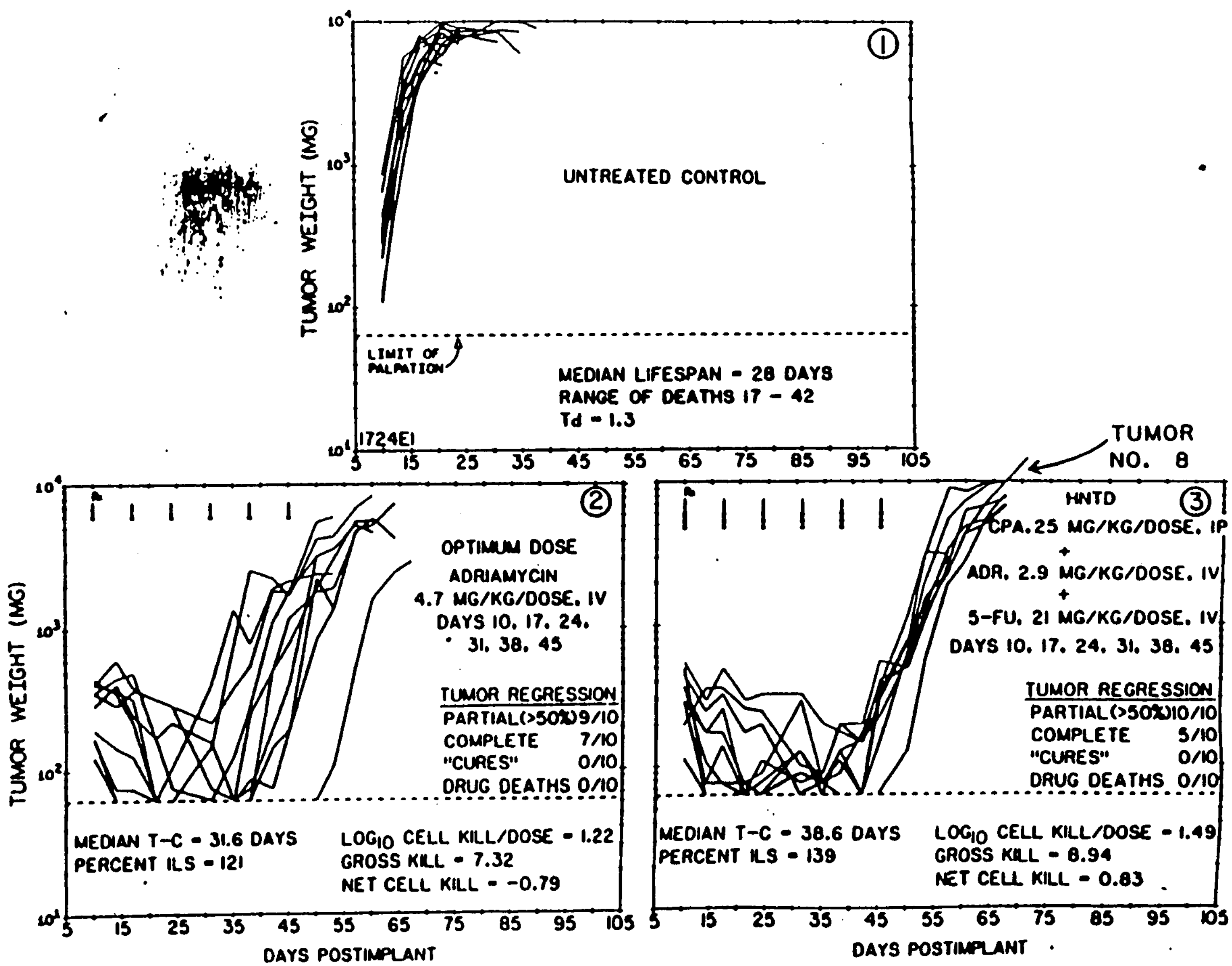

FIGURE 1.—Individual tumor response of sc implanted mammary adenocarcinoma 16/C in $B6C3F_1$ mice during treatment with ADR alone or with CPA + ADR + 5-FU. *Panel 1*—untreated control. *Panel 2*—treated with ADR, q7d × 6, starting on Day 10 postimplant. *Panel 3*—treated with CPA + ADR + 5-FU, q7d × 6, starting on Day 10 after implant. HNTD = Highest nontoxic dose from a dose-response study included as part of this experiment. Individual tumor weights were plotted until death from tumor (except No. 8).

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C3F$_1$ mice and serially transplanted through six addi- ıal passages without drug treatment. The seventh pas- e in B6C3F$_1$ mice was treated in parallel (in separate :e) with the parent tumor (not previously treated with g). The drug-resistant tumor selected by CAF (fig 1, :el 3, Tumor 8) was resistant to ADR but showed little ıo resistance to either CPA or 5-FU (data not shown).
·hen B6C3F$_1$ mice were implanted sc bilaterally[7] and ıted with ADR, the ADR-sensitive tumor failed to ap- r (fig 2, panel 3) before the ADR-resistant tumor grew ı lethal body burden (fig 2, panel 4). At a lower drug e of ADR, some of the ADR-sensitive tumors grew (fig ıanel 5) but all of the ADR-resistant tumors grew to ble size and overgrew the ADR-sensitive tumors in same host mice (fig 2, panel 6). In a separate experi- ıt, the same ADR-resistant 16/C and ADR-sensitive ent tumors were similarly bilaterally implanted in :3F$_1$ mice and treated with VCR (fig 3). The ADR-re- ant tumor overgrew and killed the mice (fig 3, panels ıd 6), while the ADR-sensitive tumor was markedly bited by treatment with VCR. We interpret these ı to indicate that spontaneous mutation to resistance .DR is accompanied by marked resistance to VCR in a or subline that had never been previously exposed to ?. This is a convincing example of likely pleiotropic tiple drug resistance in an advanced murine mam- y tumor that had never been exposed to one of the gs (VCR) to which it is now resistant.
) our knowledge, cross-resistance of this kind has ı observed and reported only once before. Kaye and en (20) have reported that Ridgway osteogenic sar- a, selected for resistance to Act D under treatment ı Act D, was cross-resistant to both ADR and VCR not to CPA. Therefore, this type of pleiotropic drug stance is obviously not unique to murine leukemias or ımary adenocarcinoma 16/C and we should expect to t again.

ılateral Cross-Resistance

Ve have previously reported that L1210/L-PAM and 38/L-PAM show marked cross-resistance to treatment :h DDPt, but L1210/DDPt and P388/DDPt retain ›ut the same sensitivity to treatment with L-PAM as he parent L1210/0 and P388/0 (21) (tables 4 and 5). nors (22) had previously reported that a line of Walk- 56 carcinosarcoma in rats, selected for resistance to \M, was completely cross-resistant to treatment with 't, but whether or not Walker 256 resistant to DDPt respond to treatment with L-PAM has not been re- ed.
ıother example of unilateral cross-resistance is vn in table 5. P388/L-PAM is cross-resistant to treatment with VCR, but P388/VCR retains full sensitivity to treatment with L-PAM.

These unilateral cross-resistances are not understood, but they could be important in drug selection for phase I-II trials or combination chemotherapy studies.

Use of Drug-Resistant Tumor Cells in New Drug Development

The 1-deaza-7, 8-dihydropteridines (listed under mitotic inhibitors in table 5: NSC-181928, NSC-269416, and NSC-330770) are of great interest because of their antitumor activity against P388/0, P388/VCR, and P388/MTX. The first compound in this series was prepared as an intermediate in the synthesis of 1-deaza-MTX (23). It was highly cytotoxic against KB cells in culture, but had very limited and equivocal cytostatic activity against L1210 in vivo. It was less active than MTX in inhibiting DHFR. NSC-181928, NSC-269416, and NSC-330770 were then prepared on the basis of their in vitro cytotoxic activity. They did not inhibit DHFR and their in vitro cytotoxicity was not reversed by folinic acid (24). They were observed to reduce the body burden of P388 stem cells by 4-5 $\log_{10}$. In studying their mechanism(s) of action, they were found to compete with colchicine for its binding sites on tubulin,[8] and VCR-resistant P388 cells showed marked sensitivity to them. Additionally, they are markedly active against P388/MTX in mice. Because of these activities against P388/0, P388/MTX, and P388/VCR, the following indications for testing in man appear plausible: (*a*) trial against MTX-sensitive tumors, particularly if resistance to MTX may be involved in ultimate treatment failure with MTX against initially MTX-sensitive tumors; (*b*) trial in combination with VCR, eg, against choriocarcinoma where MTX is useful and often curative when used alone, but where vinca alkaloids increase the therapeutic effectiveness of drug treatment when used with MTX or as second-generation treatment; and (*c*) trial in childhood ALL where both VCR and MTX are used in remission induction and maintenance therapy, and where, because of the size of the body burden of tumor stem cells at start of treatment, the overgrowth of tumor stem cells resistant to either VCR or MTX or both may be expected. Activity against both MTX- and VCR-resistant tumor cells appears to be unique, particularly with this type of structural and functional class of drugs.

DISCUSSION

Drug-resistant sublines of transplantable murine leukemias and solid tumors, with which significant and therapeutically useful reduction of the body burden of tumor stem cells can be obtained by treatment with < LD10 dos-

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ıe drug-resistant tumor was implanted sc on the right lateral thorax. he parent drug-sensitive tumor was implanted sc on the left lateral ı of each mouse.

[8] G. P. Wheeler, personal communication.

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es of representatives of all of the major chemical and functional classes of clinically useful anticancer drugs, have been isolated. Resistance, cross-resistance, and collateral sensitivity of the drug-resistant murine leukemias have been determined. Certain patterns of drug responses of these tumors appear to be consistent, and these should be useful in providing objective indications for patient selection for phase I-II clinical trials. They could also serve as guides for drug selection for clinical chemotherapy protocols where failure of drug treatment due to likely overgrowth of drug-resistant cells in initially drug-responsive tumors has occurred.

With the exception of a group of large polycyclic anticancer agents with which pleiotropic drug resistance is known to occur, mutations to drug resistance within individual chemical and functional classes of anticancer drugs are characterized by cross-resistance to very closely related drugs, particularly if the relationship is func-

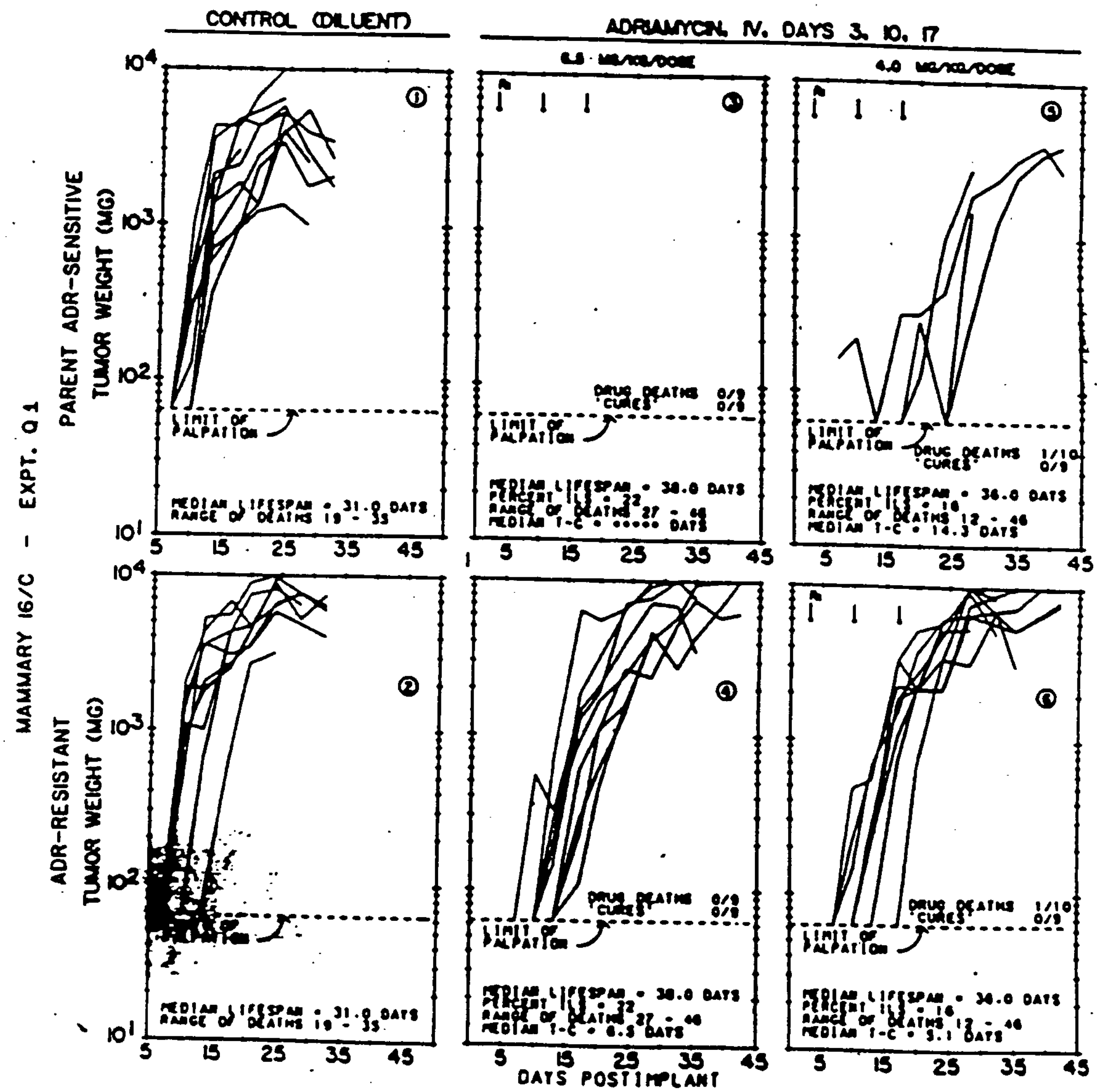

FIGURE 2.—Individual tumor responses of bilaterally implanted mammary adenocarcinoma 16/C (parent ADR-sensitive, panels 3 and 5; and ADR-resistant subline, panels 4 and 6) in B6C3F$_1$ mice during and following treatment with ADR, q7d × 3, starting 3 days after implant. Untreated control tumors plotted in panels 1 and 2. Individual tumor weights were plotted until death from tumor.

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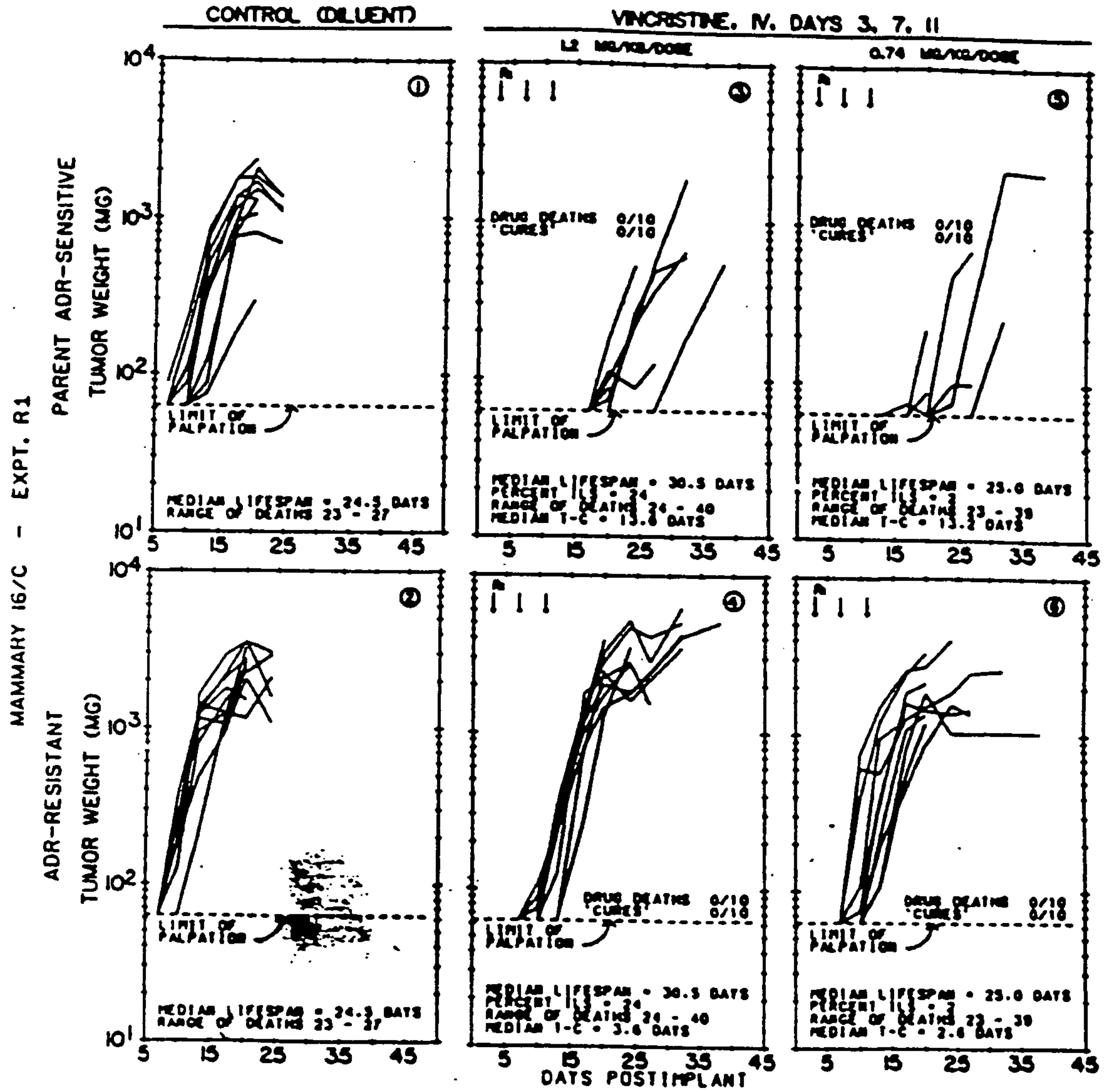
ional and not structural, although many, often not understood, exceptions to this do occur.

Within general functional classes of anticancer agents, g, alkylating agents, cross-resistance is closely related to he presence or absence of similar or identical functional noieties (5).

In addition to the obvious promise that utilization of ata from resistance, cross-resistance, and CS studies with drug-resistant murine tumors may aid in improving drug selection for treatment of cancer patients, the promise of drug-resistant tumor cells to serve as laboratory tools for increasing our understanding of both biologic and biochemical mechanism of action of anticancer drugs as well as the mechanisms of resistance, cross-resistance, and CS is obvious and already well-established.



RE 3.—Individual tumor responses of bilaterally implanted mammary adenocarcinoma 16/C (parent ADR-sensitive, panels 3 and 5, and ADR-resist. t, panels 4 and 6) in B6C3F$_1$ mice during and following treatment with VCR, q7d × 3, starting 3 days after implant. Untreated control tumors plot- l in panels 1 and 2. Individual tumor weights were plotted until death from tumor.

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APPENDIX IV

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INSTRUCTION 271B
SUMMARY OF THE USUAL CHARACTERISTICS OF[1]
SELECTED MURINE MODELS USED UNDER THE AUSPICES OF
THE NCI DIVISION OF CANCER TREATMENT (5, 6)
APRIL 1, 1978

MODEL	TUMOR	HOST: PROPAGATION	HOST: TESTING	TEST INOCULUM	CONTROL	FINAL EVALUATION DAY	PARAMETER	ACCEPTABLE CONTROL RANGE	POSITIVE CONTROL COMPOUND	TEST DATA	MODEL
[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]

Code	Tumor system	Host	Inoculum	Inoculum (treatment)	Evaluation	Parameter	Schedule
[illegible]	[illegible] lymphoid leukemia	[illegible]	[illegible]	WHOLE CELLS	[illegible]	[illegible]	[illegible] DAYS
[illegible]	[illegible] lymphoid leukemia	[illegible]	[illegible]	WHOLE CELLS	[illegible]	[illegible]	[illegible] DAYS
[illegible]	[illegible] lung [illegible]	[illegible]	FRAGMENT	FRAGMENT	[illegible]	[illegible]	NA
[illegible]	[illegible] lung [illegible]	[illegible]	FRAGMENT	FRAGMENT	VARIABLE	RELATIVE MEAN [illegible] OR REGRESSION	NA
[illegible]	[illegible] lung carcinoma	[illegible]	FRAGMENT	FRAGMENT	[illegible]	[illegible] S.T.	[illegible] DAYS
[illegible]	[illegible] lung carcinoma	[illegible]	FRAGMENT	WHOLE CELLS	[illegible]	[illegible] S.T.	NA
[illegible]	[illegible] breast [illegible]	[illegible]	FRAGMENT	FRAGMENT	[illegible]	TUMOR DIAMETER CHANGE	NA
[illegible]	[illegible] breast [illegible]	[illegible]	FRAGMENT	FRAGMENT	VARIABLE	RELATIVE MEAN [illegible] OR REGRESSION	NA
[illegible]	[illegible] lung carcinoma	[illegible]	CELLS	WHOLE CELLS	[illegible]	[illegible] S.T.	[illegible] DAYS
[illegible]	[illegible]	[illegible]	FRAGMENT	FRAGMENT	[illegible]	[illegible] S.T.	[illegible] DAYS
[illegible]	[illegible]	[illegible]	FRAGMENT	FRAGMENT	[illegible]	[illegible]	[illegible]
[illegible]	[illegible] lymphocytic leukemia	[illegible]	CELLS	WHOLE CELLS	[illegible]	[illegible] S.T.	[illegible] DAYS
[illegible]	[illegible] lymphocytic leukemia	[illegible]	CELLS	WHOLE CELLS	[illegible]	[illegible] S.T.	[illegible] DAYS
[illegible]	[illegible] mammary [illegible]	[illegible]	FRAGMENT	FRAGMENT	[illegible]	[illegible]	[illegible]

(1) CODES FOR DATA MAY BE FOUND IN INSTRUCTION [illegible]
(2) TREATMENT STARTS WHEN TUMOR REACHES WEIGHT OF [illegible]
(3) ALL CELL COUNTS ARE MADE [illegible] PROCEDURES OR OTHER METHOD APPROVED BY [illegible] PROJECT OFFICER
(4) [illegible] WEIGHT CHANGE DIFFERENCE CUTOFF; [illegible]
- [illegible] NEGATIVE SURVIVAL TEST;
- FALSE ACTIVITY IN A TUMOR WEIGHT [illegible]
(5) THE INFORMATION ON THE MOST RECENT REVISION OF THIS CHART TAKES PRECEDENCE OVER PREVIOUS INSTRUCTIONS
(6) THE NEXT REVISION OF THIS CHART IS IN PREPARATION. SEND YOUR SUGGESTIONS TO YOUR [illegible] PROJECT OFFICER
(7) [illegible] CAPSULE

INSTRUCTION [illegible] CAN BE REQUESTED FROM: [illegible] INFORMATION SECTION, DRUG EVALUATION BRANCH, DEVELOPMENTAL THERAPEUTICS PROGRAM, DIVISION OF CANCER TREATMENT, NATIONAL CANCER INSTITUTE, [illegible] ROAD, [illegible] BUILDING, ROOM [illegible], [illegible] SPRING, MARYLAND [illegible]

NA - NOT APPLICABLE
[illegible] - NOT ESTABLISHED

*[illegible] IS A NATURAL PRODUCT

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APPENDIX V

[CANCER RESEARCH 44, 717-726, February 1984]

Induction and Chemotherapeutic Response of Two Transplantable Ductal Adenocarcinomas of the Pancreas in C57BL/6 Mice[1]

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Division of Oncology, Department of Medicine, Wayne State University School of Medicine and the Michigan Cancer Foundation, Detroit, Michigan 48201 [T. H. C.]; Southern Research Institute, South Birmingham, Alabama 35255 [B. J. R., W. R. L., L. J. W., D. P. G., Jr., F. M. S., Jr.]; and Department of Toxicology, Allied Corporation, Morristown, New Jersey 07960 [J. C. P.]

ABSTRACT

Following implant of cotton thread-carrying 3-methyl-cholanthrene into the pancreas tissue of 90 C57BL/6 and 60 BALB/c mice, 13 developed ductal adenocarcinomas. Two of these tumors, both of C57BL/6 origin (Panc 02 and 03), were established in serial s.c. transplant. Panc 02 was treated with 37 different anticancer drugs representing all of the chemical and functional classes of clinically useful anticancer agents including alkylating agents, antimetabolites, agents that bind to or cause scission of DNA, and others that inhibit mitosis or inhibit protein synthesis. When drug treatment was started within 3 to 4 days after tumor implant, Panc 02 showed only limited response to treatment with two nitrosoureas, [*N'*-[(4-amino-2-methyl-5-pyrimidinyl)methyl]-*N*-(2-chloroethyl)-*N*-nitrosourea, monohydrochloride and *N*-(2-chloroethyl)-*N'*-(2,6-dioxo-3-piperdinyl)-*N*-nitrosourea)], and *N*-phosphonacetyl-L-aspartate. Drug response of Panc 03 was determined only with Adriamycin, 5-fluorouracil, cyclophosphamide, *cis*-(SP-4-2)-diamminedichloroplatinum, or *N,N'*-bis(2-chloroethyl)-*N*-nitrosourea. When drug treatment was started 3 days after tumor implant, high cure rates were obtained with Adriamycin treatment, and limited therapeutic responses were seen to treatment with *cis*-diamminedichloroplatinum or cyclophosphamide.

A comparison of the biological characteristics and drug responsiveness of Panc 02 and Panc 03 with those of a number of other transplantable tumors of mice is reported.

INTRODUCTION

Prior to the development of the tumors reported herein, there have been no transplantable pancreatic ductal adenocarcinomas of mice available for chemotherapy, radiotherapy, biochemical, or biological studies. The *in vivo* use of transplantable tumors (pancreas or other) of hamsters or rats for most chemotherapy studies is less desirable than using mouse tumors because of space requirements, higher [illegible] costs, and limited supplies of many investigational agents required for use of these larger animals. It is for these reasons that we undertook a program to chemically induce pancreatic tumors in inbred strains of mice.

Two of the 13 pancreatic ductal adenocarcinomas induced were successfully established in passage and studied for biological and drug response characteristics.

MATERIALS AND METHODS

Tumor Induction. 3-MCA[5] (500 mg) was added to a heated (~100°) solution of paraffin (3 g) in sesame oil (4 ml). The temperature was slowly increased until a solution was effected. Six-inch lengths of Coats & Clark cotton quilting thread (available in one size only) were soaked for approximately 2 min in the hot 3-MCA solution, were removed, and were allowed to cool. Loose 3-MCA was scraped from the thread. The mice [female C57BL/6 (Laboratory Supply Co., Indianapolis, IN, and Simonsen Laboratories, Gilroy, CA), male C57BL/6 (Southern Animal Farms), and female BALB/c (Harlan Industries and ARS/Sprague-Dawley, Madison, WI)] were anesthetized with pentobarbital (60 mg/kg) and laparotomized to expose the pancreas. The cotton thread was then sewn into the pancreas (one pass through the pancreas only), knotted, and trimmed. The mice were palpated and weighed weekly starting approximately 4 months postimplantation of the 3-MCA thread.

Tumor Passage. All tumors used were maintained in serial passage in the host of origin exclusively. Chemotherapy trials were carried out in an F_1 hybrid of the host of origin, *i.e.*, the tumor was transplanted from the host of origin strain into F_1 hybrid mice of that strain for the chemotherapy trials.

Chemotherapy. The techniques of chemotherapy and data analysis have been presented in detail elsewhere (4, 7, 8, 9, 17, 18). Briefly stated, the following method was used. The animals necessary to begin an experiment were pooled, implanted s.c. with 30- to 60-mg tumor fragments by trocar, and again pooled before unselective distribution to the various treatment and control groups. Chemotherapy was started within 1 to 5 days after tumor implantation, while the number of cells was relatively small (10^7 to 10^8 cells; early-stage disease). Tumors were measured with a caliper twice weekly until the death of the animal or cure was assured. Tumor weights were estimated from 2-dimensional measurements:

$$\text{Tumor wt (mg)} = (a \times b^2)/2$$

where a and b are the tumor length and width (mm), respectively.

End Points for Assessing Antitumor Activity. The following quantitative end points were used to assess antitumor activity:

(*a*) Percentage of increase in host life span = 100 × [(MDD of the treated tumor-bearing mice) − (MDD of the tumor-bearing control mice)/MDD of the tumor-bearing control mice]

and (*b*) the T-C value, where T and C were the median time (days)

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[5] The abbreviations used are: 3-MCA, 3-methylcholanthrene; MDD, median day of death; TD, tumor volume doubling time; LD_{10}, dosage that caused lethality in 10% of mice; T-C, tumor growth delay; ROS, Ridgway osteogenic sarcoma; 5-FU, 5-fluorouracil (NSC 19893); cis-DDPt, (SP-4-2)-diamminedichloroplatinum (NSC 119875); ADR, Adriamycin (NSC 123127).

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required for the treatment group and the control group tumors, respectively, to reach a predetermined size (500 or 750 mg). Tumor-free survivors were excluded from these calculations. In our judgment, this value was the single most important criterion of antitumor effectiveness because it allowed the quantitation of tumor cell kill.

Calculation of Tumor Cell Kill. For s.c.-growing tumors, the $\log_{10}$ cell kill per dose was calculated from the following:

$$\text{Log}_{10} \text{ kill per dose} = \frac{\text{T-C value in days}}{(3.32)\ (\text{TD})\ (\text{no. of doses})}$$

where TD (in days) was estimated from the best-fit straight line from a log-linear growth plot of the control group tumors in exponential growth (100- to 800-mg range). The conversion of the T-C values to $\log_{10}$ cell kill was possible because the TD of tumors regrowing posttreatment approximated the TD values of the tumors in untreated control mice.

$$\text{Log}_{10} \text{ cell kill ((gross or total)} = \frac{\text{T-C value in days}}{(3.32)\ (\text{TD})}$$

$$\text{Log}_{10} \text{ cell kill (net)} = \frac{(\text{T-C value in days}) - (\text{duration of treatment in days})}{(3.32)\ (\text{TD})}$$

If the $\log_{10}$ cell kill (net) value was positive, there were fewer cells present at the end of therapy than at the start. If, on the other hand, the value was negative, the tumor grew under treatment. A positive gross value with a negative net value indicated inhibition of growth of the tumor cell population during drug treatment.

The $\log_{10}$ kill values were converted to an arbitrary activity rating published previously (Table 1) (8).

It has been our experience that, if this conversion is not used, a single injection will invariably appear superior to longer treatment regimens when net cell kills are compared. Likewise, therapies of >20 days will appear superior to single injection schedules if gross tumor cell kills are evaluated and compared (Table 1). No agent received a ++++ activity rating unless a 40% or greater percentage of increase in host life span value was also obtained. An activity rating of +++ or ++++ is needed to effect partial or complete regressions of 100- to 300-mg masses of most transplanted solid tumors of mice (8, 9). Thus, an activity rating of + or ++ would not be scored as active by usual clinical criteria.

The growth and chemotherapeutic response behavior of 6 transplantable tumors of mice were used for comparison with the 2 transplantable pancreatic ductal adenocarcinomas. The induction (or discovery) and drug response characteristics of the following tumors have been described: Colon 36, 51, and 26 (3, 4, 7, 8, 17, 18); Mammary 16/c (5, 8, 9, 17); ROS (15, 17, 24); ^{239}Pu-induced osteogenic sarcoma (8, 12). The ^{239}Pu-induced osteogenic sarcoma was obtained as a cell culture suspension from Dr. L. A. Glasgow. It was readily reestablished *in vivo* following s.c. implants of the cultured cells in C57BL/6 mice.

RESULTS

Tumor Induction and Tumor Biology. The induction of pancreatic ductal adenocarcinomas by the implantation of cotton

Table 1

Comparison of $\log_{10}$ cell kill values to an activity rating

	Duration of treatment <5 days $\log_{10}$ kill	Duration of treatment 5 to 20 days $\log_{10}$ kill		Duration of treatment >20 days $\log_{10}$ kill	
Activity rating[a]	Net	Net	Gross	Net	Gross
++++	>2.8	>2.0	>2.8	>0.8	>3.4
+++	1.6–2.8	0.8–2.0	2.0–2.8		2.5–3.4
++	0.9–1.5		1.3–1.9		1.7–2.4
+	0.5–0.8		0.7–1.2		1.0–1.6
–	<0.5		<0.7		<1.0

[a] Where ++++ is highly active, and – is inactive.

Table 2

Tumors induced in the pancreas of BALB/c and C57BL/6 mice by the implantation of cotton threads saturated with 3-MCA

Data are pooled from 2 separate experiments with female C57BL/6 and female BALB/c mice.

Mouse strain (sex)	No. of mice surviving thread implantation	No. of mice alive: 120 days	240 days	360 days	480 days	600 days	No. of mice that died, not examined for tumors	No. of mice sacrificed, examined for tumors[a]	No. of tumors found in sacrificed mice	No. of tumors of each type found in sacrificed mice: Pancreatic ductal adenomas, hyperplasia (days of latency)[b]	Mixed tumor-ductal cells and mesenchymal elements[c] (days of latency)	Fibrosarcomas (days of latency)	Others (days of latency)	Pancreatic ductal adenocarcinomas (days of latency)
C57BL/6 (F)	67	61	38	23	7	5	15	52	40	4 (74, 117, 243, 270)	9 (98, 146, 146, 154, 179, 179, 186, 420, 636)	19 (117, 123, 123, 128, 130, 137, 154, 165, 179, 189, 198, 221, 235, 236, 243, 243, 270, 270, 333)	0	8 (270, 288, 445, 445, 445, 460, 473, 706)
C57BL/6 (M)	23	17	13	10	8	0	7	16	7	0	0	6 (99, 99, 99, 137, 137, 331)	0	1 (528)
BALB/c (F)	60	48	33	20	15	2	24	36	21	6 (83, 102, 222, 222, 256, 490)	1 (102)	8 (92, 171, 172, 190, 253, 256, 446, 563)	2 (102[d], 256[e])	4 (222, 222, 431, 807)

[a] Starting 4 months postimplant of the carcinogen, mice were palpated weekly and sacrificed if a mass was detected. [b] Days of latency, from the time of 3-MCA implant until the tumors were transplanted. Tumor sizes at transplant varied from 0.1 to 4 g. [c] Carcinosarcomas. [d] Squamous cell carcinoma. [e] Undifferentiated carcinoma.

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threads saturated with 3-MCA was attempted on 60 BALB/c and 90 C57BL/6 mice. The tumors that arose with short latency periods (<220 days) were usually fibrosarcomas or tumors containing sarcomatous elements. Those tumors that arose with the longer latency periods were frequently pancreatic ductal adenocarcinomas (Table 2). Indeed, no pancreatic ductal adenocarcinomas arose before 220 days postimplantation of the carcinogen, and only 15 of 43 of the fibrosarcomas and mixed tumors arose after that period. Two of the 13 pancreatic ductal adenocarcinomas were established in serial passage (Panc 02, latency 528 days; and Panc 03, latency 473 days). The other 11 adenocarcinomas were transplanted but failed to survive the first passage. The biological characteristics of Panc 02 and 03 are listed in Table 3 and compared with 6 other transplantable solid tumors of mice. Photomicrographs of Panc 02 and Panc 03 are shown in Figs. 1 to 4.

Panc 02 originated as a Grade II tumor (Grade IV being undifferentiated), producing copious amounts of fluid and ulcerating through the skin after trocar implant (without infection or necrosis) at a very small size (<400 mg). The tumor also carried a benign connective tissue component. Given the early surface ulceration and fluid production properties, the tumor was unsuitable for chemotherapy trials. At passage 26, the tumor was established in cell culture by methods described previously (25). After transplantation back into mice, the tumor retained a well-differentiated histological appearance (a Grade III tumor) but produced very little fluid, did not ulcerate to the surface at a small size, and contained no connective tissue elements. All chemotherapy trials were carried out in mice on the line passaged in cell culture. We found Panc 02 to be among the most metastatic solid tumors evaluated to date (gross metastases were seen in the lungs of >70% of all tumor deaths). Surgical removal of 500- to 900-mg s.c. tumors (15 days postimplant of 30- to 60-mg fragments; 29th passage) resulted in only one cure in 15 mice. Metastases were noted in the lungs, lymph nodes, and kidneys. No postsurgical primary site regrowths occurred.

Panc 03 also originated as a Grade II tumor, producing fluid in variable quantities and also ulcerating to the surface, although usually at sizes >800 mg. The tumor was suitable for chemotherapy trials. No attempt was made to establish Panc 03 in cell culture. The metastatic behavior of Panc 03 remains to be determined at a size suitable for surgical removal (500 to 1500 mg), although gross metastases in the lungs were seen in only 5 of 28 mice dying from large s.c. tumor masses.

Chemotherapy. Panc 02 at an early stage of development (30- to 60-mg size) was examined for therapeutic responsiveness to 37 anticancer agents. These agents were used by schedules and routes of administration known to be active against other transplantable mouse tumors, [Except for tubercidin (NSC 56408), which was found to be inactive against all transplanted solid tumors evaluated to date]. A minimum of 3 dosage levels (usually 10 mice/group) were evaluated (~1.5, 1.0, and 0.67 × historic LD_{10} values). In all cases, the highest dose was toxic ($\geq LD_{20}$), establishing adequate treatment. Tumor growth plots of Panc 02 treated with 5-FU (NSC 19893) and ADR are shown as typical examples (Charts 1 and 2). The highest nontoxic dosage ($\leq LD_{10}$) was evaluated for antitumor activity (Table 4). At the highest nontoxic dosage (LD_{10} or less), none of the agents evaluated was considered to be even moderately active (+++ activity rating, the minimum degree of cell killing needed to effect partial regressions of most transplantable solid tumors of mice). Three agents were weakly active (+ activity rating): 2 nitrosou-

Table 3

Biological characteristics of 2 transplantable ductal adenocarcinomas of the pancreas compared with 6 other transplantable tumors of mice

Tumor	Mouse of origin	Carcinogen	Date of original transplant	% of metastases to lungs from 1000-mg s.c. tumor	Histology	Grade	Days for s.c. mass to reach 500 mg after trocar implant of 50-mg fragments (median at current generation)	Days of approximate recent generation tumor volume doubling time (100–800 mg)
Panc 02	C57BL/6[a]	3-MCA	7/28/78[b]	>80	Adenocarcinoma	III[c]	9–17 (12)	2.1–4.2
Panc 03	C57BL/6[d]	3-MCA	12/1/81[e]	Unknown	Adenocarcinoma	II	20–43 (25)	4.5–8.1
Colon 38	BALB/c[d]	1,2-dimethyl hydrazine	6/21/73	<5	Adenocarcinoma	III	18–29 (20)	3.1–5.0
Colon 51	BALB/c	1,2-dimethyl hydrazine	6/6/73	>80	Adenocarcinoma	III	13–24 (17)	2.2–6.3
Colon 26	BALB/c	Nitrosomethyl urethan	6/5/73	>80	Undifferentiated carcinoma	IV[f]	11–16 (14)	2.0–3.0
Mamm 16/c	C3H/He[d]	Spont. (virus assoc.)	1974	>80	Adenocarcinoma	III	7–11 (8)	1.2–2.0
^{239}Pu-induced Ost Sar	C57BL/6	^{239}Pu	1976	~10	Undifferentiated sarcoma	IV	7–10 (9)	1.2–1.9
ROS	AKm[a]	Spont.	11/18/48	<1	Undifferentiated sarcoma	IV	11–15 (13)	1.4–2.0

[a] Male mouse.
[b] Latency, 528 days; current passage generation is number 72 (March 1983).
[c] Becoming more undifferentiated with continuous passage.
[d] Female mouse.
[e] Latency, 473 days; current passage generation is number 9 (March 1983).
[f] IV, undifferentiated.

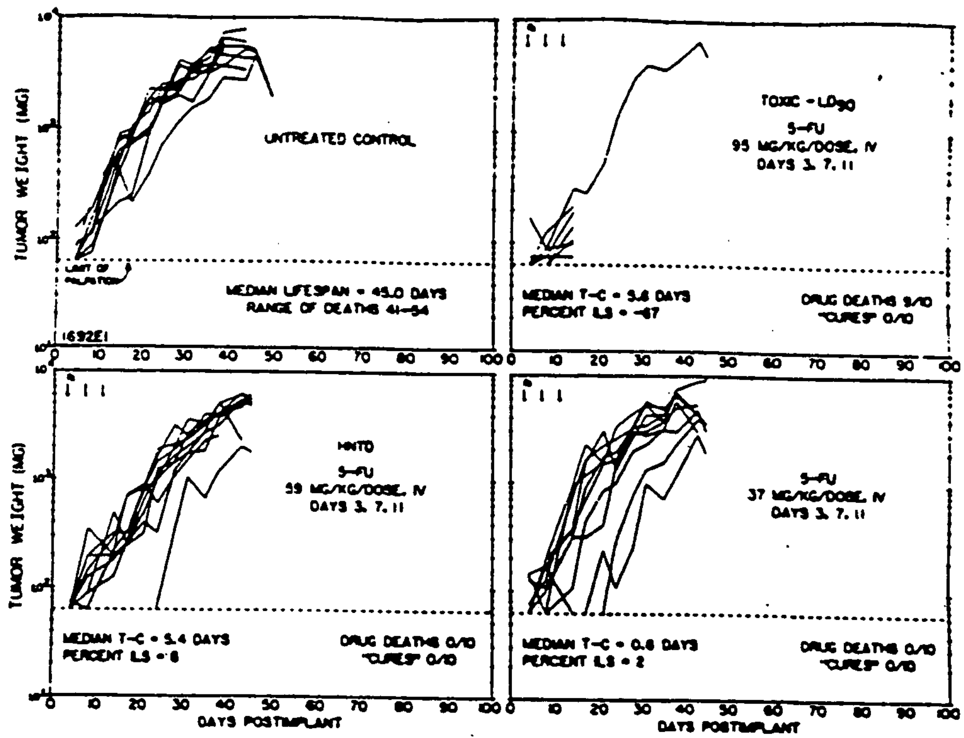

Chart 1. 5-FU treatment of pancreas carcinoma 02. For experimental method, see Table 3 and "Materials and Methods." Individual tumor growth was plotted for 3 dosage levels. No significant tumor growth delay was noted at the highest nontoxic dose (*HNTD*). *Percent ILS*, percentage of increase in host life span.

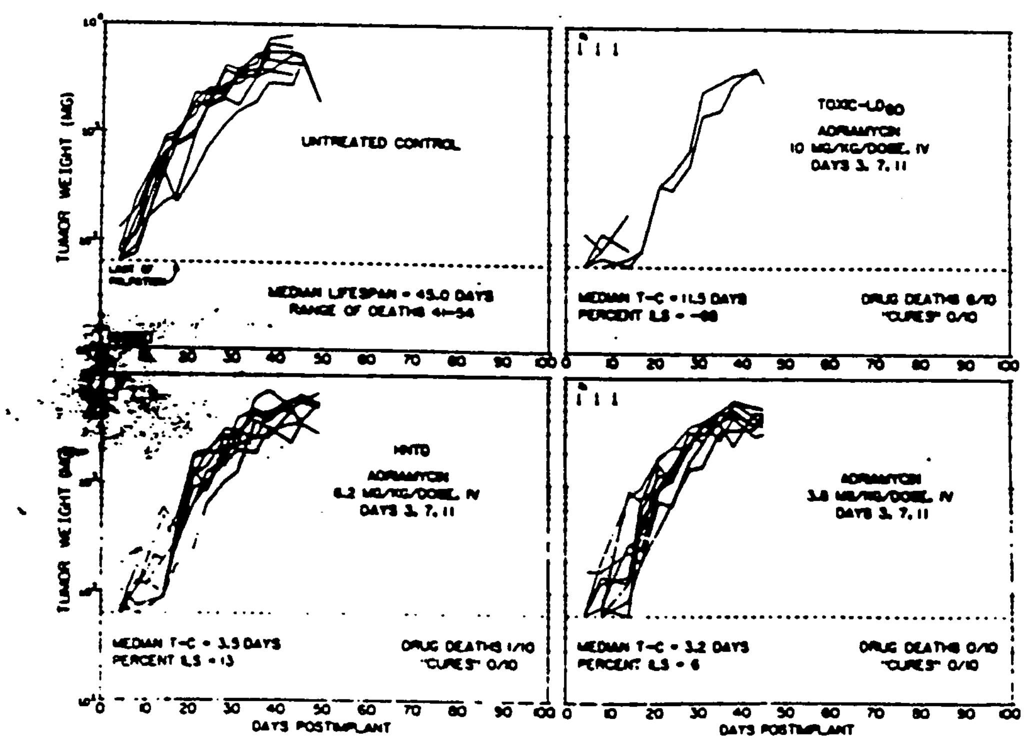

Chart 2. ADR treatment of pancreas carcinoma 02. For experimental method, see Chart 1. *Percent ILS*, percentage of increase in host life span.

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reas, N'-[(4-amino-2-methyl-5-pyrimidinyl)methyl]-N-(2-chloroethyl)-N-nitrosourea, (NSC 245382) and N-(2-chloroethyl)-N'-(2,6-dioxo-3-piperdinyl)-N-nitrosourea (NSC 95466); and N-phosphonacetyl-L-aspartate (NSC 224131) (Table 4).

The insensitivity of this highly metastatic tumor to the broad range of chemotherapeutic agents was obviously of great interest, since it mimicked the majority of human tumors of this type. We considered the possibility that the unresponsiveness may have been the result of passage of the tumor through cell culture. Although this possibility cannot be totally ruled out for this particular tumor, many other tumors retain marked responsiveness to antiproliferative agents after passage in culture. For example, all *in vivo* chemotherapy trials with the ^{239}Pu-induced osteogenic sarcoma (listed in Table 6) were carried out on a tumor derived from a cell culture line (12). We also maintained a cell culture line of Colon 26 that retained marked responsiveness to the nitrosoureas and moderate responsiveness to 5-FU when tested in mice.[6]

We also considered the possibility that the tissue of origin may have been responsible for the broad insensitivity of Panc 02. It is well known that many tumors arising from certain tissues have a high degree of responsiveness to selected agents. Examples are numerous: Wilm's tumor to actinomycin D, Hodgkin's disease to procarbazine, breast tumors to ADR, acute myeloblastic leukemia to 1-β-D-arabinofuranosylcytosine, testicular cancer to *cis*-DDPt, B-cell leukemias to many of the available antitumor agents, etc. It is also well known that many tumors retain differentiated features of the tissue of origin and, thus, may retain drug sensitivities of the tissue origin (or alternately acquire a particular sensitivity because of the particular state of differentiation at the time the cancer conversion took place. We considered the possibility that the opposite could also occur, *i.e.*, generalized insensitivity could be related to the intrinsic properties of the tissue of origin. Thus, one would project that the other transplantable pancreatic tumor would also be equally drug insensitive. Panc 03 has only recently been passed *in vivo* for a sufficient number of generations to establish a reproducible take-rate and the stable growth behavior necessary for objectively reliable chemotherapy trials. The results with the first 5 agents evaluated provided a definitive answer to the issue. Panc 03, which is slower growing and less metastatic than Panc 02, was markedly sensitive to ADR (8 of 10 cures of early-stage disease and a 3.3 $\log_{10}$ tumor cell kill among those 2 tumors not cured), and modestly responsive to *cis*-DDPt and carboxypeptidase A (1.1 and 1.8 $\log_{10}$ cell kill, respectively) (Table 5). Neither 5-FU nor N,N'-bis(2-chloroethyl)-N-nitrosourea was active (Table 5).

DISCUSSION

The discovery of these 2 transplantable pancreatic ductal adenocarcinomas of mice may provide experimental tumor models that can aid biological, biochemical, radiotherapeutic, and chemotherapeutic studies of this generally unresponsive cancer of humans. Although the testing with Panc 03 was limited, the marked responsiveness of this tumor to ADR and the moderate responsiveness to *cis*-DDPt and cyclophosphamide may provide a rationale for a clinical trial with the potentiating combinations of ADR + *cis*-DDPt (2, 20) or ADR + *cis*-DDPt + cyclophosphamide (11). Based on the relative activities of these agents against Panc 03, a dosage ratio containing the highest proportion of ADR would be favored for combination usage (9, 22, 23). Enthusiasm for the chemotherapeutic treatment of pancreatic cancer with currently available agents is, however, tempered by the results obtained with Panc 02: a tumor model that seems to mimic the modest to poor results of many clinical trials in humans (10, 13, 14, 26, 27).

The finding of a tumor (Panc 02) that is intrinsically insensitive to 34 different antitumor agents and only weakly responsive to 3 others is perhaps not completely unexpected if one considers the general patterns of antitumor drug responses in other transplantable solid tumors of mice. It has been recognized for many years that, in most cases, there are clear differences between resistance and innate insensitivity to an antitumor agent (6, 21). In the first case, the tumor responds to treatment, often undergoing a prolonged remission or regression, only to eventually regrow in the face of the same continuing drug therapy. This regrowth is usually due to cells specifically resistant, either partially or completely, to the drug (19). It is generally accepted that tumor stem cells specifically resistant to any drug and not induced by drug treatment are likely to be present (one of 10^4 to 10^6) in the primary tumor (19). In the second case, the tumor that is intrinsically insensitive to the agent will continue to grow, without evidence of an initial response, unaffected by maximum tolerated dosages (~LD_{10}). In other words, the intrinsically insensitive tumor cells possess no more vulnerability to the antiproliferative agent than do the normal cells of the host that are responsible for the dosage limitations, *e.g.*, WBC, platelets, and growth-inhibiting epithelium. The essential feature of drug response in randomly chosen transplantable solid tumors is the absence of an orderly or predictable pattern of either vulnerabilities or intrinsic insensitivities to any given set of antiproliferative agents (although there is often an increase in the frequency of tumors from a given organ system that respond to a particular drug, *e.g.*, ~60% of transplantable breast tumors respond well to ADR).

Examples of the haphazard response patterns of several tumors are listed in Table 6. These range from among the most responsive of solid tumors (ROS) to among the most unresponsive (Colon 51 and Panc 02). Each of these tumors is intrinsically insensitive, and each (except Panc 02) is markedly sensitive to one or more of the agents listed. Reciprocal patterns of sensitivities are common among these and other tumors (4, 5). For example, Mammary 16/c is highly responsive to ADR and insensitive to N,N'-bis(2-chloroethyl)-N-nitrosourea the opposite pattern is seen with Colon 26 (highly responsive to N,N'-bis(2-chloroethyl)-N-nitrosourea and insensitive to ADR). In another example, Colon 36 is highly sensitive to 1-β-D-arabinofuranosylcytosine and insensitive to N-(2-chloroethyl)-N'-(2,6-dioxo-3-piperdinyl)-N-nitrosourea; the opposite pattern is seen with Colon 51. Interestingly, both Colon 36 and 51 were induced in the same organ with the same dose of the same carcinogen in the same birthdate batch of inbred BALB/c mice from the same supplier (3), illustrating that the specific sensitivities of tumors to currently available drugs may often be acquired unpredictably during carcinogenesis, and are clearly independent of host factors.

In isolated instances, intrinsic insensitivities to a particular antitumor agent could be due to a specific mutational event (*e.g.*,

[6] T. H. Corbett and B. J. Roberts, unpublished results.

Table 4

Response of early stage, s.c. growing pancreatic ductal adenocarcinoma 02 to 37 antitumor agents

For the experimental method, 30- to 60-mg fragments of Panc 02 were implanted s.c. by trocar into the axillary region of BDF$_1$ mice on Day 0. Drug treatment began 2 to 4 days later and was continued until 20% or more of the mice died from toxicity at the top dosage level (see "Materials and Methods").

Transplant generation	Agents	[illegible][a]	Drug route and schedule[b]	Drug deaths[c]	MDD control mice	% ILS treated mice	Tumor-free survivors: Controls	Tumor-free survivors: Treated	Exponential TD[d] (days)	Time for tumors to reach 750 mg	Median tumor growth delay (T-C in days)[e]	Log_{10} cell kill activity: Gross[d]	Log_{10} cell kill activity: Rating[f]
	Alkylating agents[e]												
32	ACNU	[illegible]	i.p., 4	0/10	43	9	0/20	2/10	3.6	17.2	6.8	0.57	+
71	BCNU	26	i.p., 2	0/6	41	5	0/6	0/6	3.9	19	3.0	0.23	–
26	Streptozotocin	121	s.c., 3, 7, 11, 15, 19, 23	0/10	44	–6	2/20[f]	0/10	4.2	17.5	6.4	0.48	–
28	PCNU	8.5	p.o., 3, 7, 11, 15	0/10	44	29	2/20[f]	1/10	4.2	17.5	10.8	0.76	+
29	cis-DDPt	4.8	i.v., 3, 7	0/10	35	–8	1/20[f]	0/10	2.8	13.0	3.0	0.32	–
34	CHIP	42	i.p., 3, 7	0/10	38	1	0/20	0/10	2.1	11.1	0.1	0	–
34	DACH	32	i.p., 3, 7, 11, 15	0/10	38	–5	0/20	0/10	2.1	11.1	0.1	0	–
27	Cyclophosphamide	76	i.v., 3, 7, 11, 15	0/10	45	20	0/20	0/10	4.0	16.2	5.7	0.44	–
32	Cyclophosphamide	330	i.p., 4	0/10	43	15	0/20	0/10	3.6	17.2	1.6	0.15	–
28	Chlorambucil	23.5	p.o., 3, 7, 11, 15	0/10	44	5	0/20	0/10	4.2	17.0	–1.0	0	–
31	L-Sarcolysin	4.5	i.v., 3, 7, 11	0/10	37	–5	0/20	0/10	2.9	13.9	1.3	0.14	–
38	AZQ	4.8	i.v., 3, 5, 7, 9	0/10	33	22	0/20	0/10	2.6	12.6	2.1	0.24	–
30	Dibromodulcitol	124	s.c., 3–12	0/10	38	6	0/20	0/10	2.9	13.2	6.7	0.70	±
26	DTIC	144	p.o., 3, 7, 11, 15, 19, 23, 27	0/10	44	13	2/20[f]	0/10	4.2	17.5	7.7	0.56	–
32	Piperazinedione	7.4	i.v., 3, 7, 11	0/10	40	7	0/20	0/10	3.6	14.6	0.0	0	–
30	Procarbazine	154	p.o., 3, 5, 7, 9, 11, 13	0/10	38	6	0/20	0/10	2.9	13.2	3.8	0.40	–
27	Mitomycin C	2.1	i.v., 3, 7, 11	1/10	45	–5	0/20	0/10	4.0	18.2	2.8	0.21	–
	DNA binders[e]												
27	ADR	6.2	i.v., 3, 7, 11	1/10	45	13	0/20	0/10	4.0	16.2	3.5	0.27	–
32	Aclacinomycin A	6.0	i.v., 3, 5, 7, 9, 11	0/10	39.5	1	0/20	1/10	3.6	14.6	2.9	0.25	
29	Actinomycin D	0.5	i.v., 3, 7, 11	1/10	35	24	1/20[f]	0/10	2.8	13.0	5.6	0.60	–
29	AMSA	9.4	i.v., 3, 5, 7	0/10	36	0	1/20[f]	1/10	2.8	13.0	2.4	0.27	–
28	Ametantrone	21	s.c., 3–17	0/10	44	20	2/20[f]	1/10	4.2	17.5	6.6	0.45	–
33	Ametantrone	15.3	i.v., 4, 6, 8, 10, 12, 14	0/10	41	2	0/20	1/10	2.7	19.5	–0.5	0	–
28	Mitoxantrone	1.6	s.c., 3–13	0/10	44	40	0/20	0/10	4.2	7.0	2.3	0.17	–
	Antimetabolites[e]												
32	AAFC	46	i.p., 3×/day,[g] 3–8	0/10	39.5	–9	0/20	0/10	3.6	14.6	–0.1	0	–
33	2-Fluoro-ara-AMP	110	i.p., 3×/day,[g] 3–15	0/10	41.5	0	0/20	0/10	3.7	18.7	1.9	0.15	–
28	PalmO-ara-C	18.7	i.p., 3–10	0/10	44	–7	2/20[f]	0/10	4.2	17.5	1.6	0.16	–
27	5-FU	58	i.v., 3, 7, 11	0/10	45	8	0/20	0/10	4.0	18.2	5.4	0.39	–
30	5-FdUrd	80	i.p., 3–7, 11–15, 19–22	0/10	38	30	0/20	0/10	2.9	13.2	9.2	0.98	–
32	Hydroxyurea	115	s.c., 3×/day,[g] 3–7	1/10	39.5	–12	0/20	0/10	3.6	14.6	–0.8	0	–
31	PALA	112	i.p., 3–12	0/10	37	43	0/20	0/10	2.9	13.9	7.5	0.78	+
28	6-thioguanine	7.8	i.p., 3, 7, 11, 15, 19	0/10	44	–38	0/20	0/10	4.2	17.0	–1.3	0	–
28	Triazine antifol	80	i.p., 3–14	1/10	44	7	2/20[f]	0/10	4.2	17.5	2.9	0.24	–
33	Tubercidin	0.66	i.p., 3×/day,[g] 4–7	2/10	41	–6	0/20	0/10	2.7	19.5	–3.0	0	–
	Others												
29	Anguidine (P)	5.9	i.p., 3–7	0/10	35	0	1/20[f]	0/10	2.8	13.0	3.3	0.35	–
30	Bleomycin (S)	2	i.p., 3×/day,[g] 3–13	0/10	38	13	0/20	0/10	2.9	13.2	6.5	0.66	–
33	Bleomycin (S)	13.4	i.v., 4, 6, 8, 10, 12, 14	0/7	41	7	0/20	1/7	2.7	19.5	2.8	0.31	–
28	Ellipticine (P)	40	i.p., 3, 7, 11, 15	0/10	44	3	0/20	0/10	4.2	17.0	–2.5	0	–
27	Vincristine (M)	2.5	i.v., 3, 7	0/10	45	0	0/20	0/10	4.0	16.2	4.3	0.32	–
38	VP16 (S M)	11.5	i.v., 3, 5, 7, 9	0/10	33	16	0/20	0/10	2.6	12.6	2.8	0.32	–

[a] HNTD, highest nontoxic dosage (LD_{10} or less). The dosage levels 1.5 to 1.6 times those listed were an LD_{20} or greater; MDD, median day of death of the diluent treated control mice; % ILS, percentage of increase in host life span; TD, tumor volume doubling time; (P), protein synthesis inhibitor; (S), DNA chain scission; (M), mitotic inhibitor (likely mechanisms of action); AAFC (NSC 166641), [2R,(2d,3B,3aB,9aB)]-7-fluoro-

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deletion of deoxycytidine kinase for 1-β-D-arabinofuranosylcytosine resistance) occurring within the first few cell doublings of the original tumor and, thus, becoming a high-frequence cell type within the tumor mass. However, mutationally related resistance to one agent (especially alkylating agents and antimetabolites) has not produced resistance to most agents of differing chemical classes and differing mechanisms of action (19). Thus, the insensitivities to several different antiproliferative agents of different chemical classes that appear to occur in all tumors (Refs. 4, 5, and 8; Table 6), are unlikely to be explained by multiple mutational events which independently occur at a frequency of only 10^{-4} to 10^{-6}. Further, any hypothetical mutation to resistance that would confer broad insensitivity to multiple classes of antiproliferative agents would seem inconsistent with the pattern of marked vulnerabilities that haphazardly occur in these same tumors (Refs. 4, 5, and 8; Table 6).

The contention that any tumor will respond to any of the antitumor drugs if treatment is initiated at a small enough size (favorable growth kinetic status) is simply not true (6). Many rapidly growing tumors like Panc 02, Colon 26, Colon 51, ^{239}Pu-induced osteogenic sarcoma, mammary adenocarcinoma 16/c, and even ROS are totally insensitive at a small size to dosages of agents that are curative for one or more of the other tumors as well as selected slower-growing tumors.

Thus, in the examination of a large number of different transplantable solid tumors of mice from various organ systems, it is clear that each has a different pattern and degree of responsiveness to currently available antitumor agents (4, 5, 8, 9, 15, 17, 24). Some are markedly vulnerable to several different agents from several chemical classes (e.g., ROS), whereas most others are markedly vulnerable to only a few and often only one agent or class of agents (e.g., Colon 51). All appear to have a few very modest responses to maximum tolerated dosages of various drugs, but these would be classified as inactive by clinical standards (partial regressions required for activity). All transplantable tumors examined appear to be intrinsically insensitive to several agents that are highly active against some other tumor.

In general, one wishes to evaluate the chemotherapeutic response characteristics of a number of transplantable tumors from a given organ system and histological type in the hopes of finding redundancies in vulnerabilities that could translate into useful single-agent and combination-agent treatments for the clinic. In selected tumor types (e.g., breast), a degree of redundancy has been found (1, 5, 16). In other cases (e.g., colorectal tumors), a more random pattern of vulnerabilities has been seen (4, 8, 9). This later case (and tumors like Panc 02) provides few clinical leads and may cause one to question the methods by which most antitumor agents are selected, *i.e.*, should we be selecting agents on the basis of the peculiar drug response characteristics (vulnerabilities) of a single uncommon tumor of mesenchymal origin (P388 leukemia)? Will not the single primary selection model (with a certain set of marked vulnerabilities) repeatedly select the same types of agents with the same mechanisms of antiproliferative activity? Is it not reasonable to assume that other tumors, especially of ectodermal or entodermal origin, may have marked vulnerabilities to agents inactive against the current selection model? Is it not possible that agents with modest activity against a true cancer target (an activity which could be improved upon with analog synthesis) may be overlooked because of the marked vulnerabilities of the primary selection model (P388) to so many antiproliferation targets?

2,3,3a,9a-tetrahydro-3-hydroxy-6-imino-6H-furo[2',3':4,5]oxazolo[3,2-a]pyrimidine-2-methanol; Aclacinomycin A (NSC 208734), Adriamycin analog; ACNU (NSC 245382), N'-[(4-amino-2-methyl-5-pyrimidinyl)methyl]-N-(2-chloroethyl)-N-nitrosourea, monohydrochloride; Actinomycin D (NSC 3053); AMSA (NSC 249992), 4'-(9-acridinylamino)-methane sulfon-m-anisidide; Anguidine (NSC 141537), (3α,4β)-12,13-epoxytrichothec-9-ene-3,4,15-triol,4,15-diacetate; Ametantrone, anthracenedione acetate (NSC 287513), 1,4-bis[[[2-[(2-hydroxyethyl)amino]ethyl]amino]]-9,10-anthracenedione,diacetate; 2-fluoro-ara-AMP (NSC 328002), adenine, 2-fluoro-9-β-D-arabinofuranosyl-,5'-phosphate, 1.8 sodium salt, 2.33 hydrate; AZQ, aziridinylbenzoquinone (NSC 182986), [2,5-bis(1-aziridinyl)-3,6-dioxo-1,4-cyclohexadiene-1,4-diyl]biscarbamic acid, diethyl ester; BCNU (NSC 409962), N,N'-bis(2-chloroethyl)-N-nitrosourea; bleomycin (NSC 125066); CHIP (NSC 256927), platinum,dichlorodihydroxybis-(2-aminopropane); chlorambucil (NSC 3088); cyclophosphamide (NSC 26271); DACH (NSC 271674), platinate(1-)[1,2,4-benzenetricarboxylato(3-)-O',O''] (1,2-diaminocyclohexane); Dibromodulcitol (NSC 104800), 1,6-dibromo-1,6-dideoxygalactitol; DTIC (NSC 45388), 5-(3,3-dimethyl-1-triazenyl)-1H-imidazole-4-carboxamide; ellipticine (NSC 71795); 5-FdUrd (NSC 27640), fluorodeoxyuridine; hydroxyurea (NSC 32065); mitomycin C (NSC 26980); Mitoxantrone, dihydroxyanthracenedione (NSC 279836), 1,4-dihydroxy-5,8-bis[[[2-[(2-hydroxyethyl)amino]ethyl]amino]]-9,10-anthracenedione; PALA (NSC 224131), N-phosphonacetyl-L-aspartate; PalmO-ara-C (NSC 135962), 4-amino-1-[5-O-(1-oxohexadecyl)-β-D-arabinofuranosyl]-2(1H)-pyrimidinone (depot form of ara-C); PCNU (NSC 95466), N-(2-chloroethyl)-N'-(2,6-dioxo-3-piperidinyl)-N-nitrosourea; piperazinedione (NSC 135758), 3,6-bis-(5-chloro-2-piperidinyl)-2,5-piperazinedione,dihydrochloride; procarbazine (NSC 77213); streptozotocin (NSC 85998); 6-thioguanine (NSC 752); Triazine antifol (NSC 127755), 3-chloro-4-[[4-[2-chloro-4-[4,6-diamino-2,2-dimethyl-s-triazin-1(2H)-yl]phenyl]butyl]]benzenesulfonyl fluoride, 1:1 with ethanesulfonic acid; tubercidin (NSC 56408); vincristine (NSC 67574); VP16 (NSC 141540).

[b] Schedule in days postimplant of [illegible]

[c] Drug-induced deaths at the [illegible] dosage (tumor-bearing mice).

[d] See "Materials and Methods."

[e] Probable mechanism of action.

[f] No-takes.

[g] Three- to 4-hr separation between injections.

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Table 5

Response of early stage, s.c. growing pancreatic ductal adenocarcinoma 03 to 5 antitumor agents

For the experimental method, 30- to 60-mg fragments of Panc 03 (ninth generation) were implanted s.c. by trocar into the axillary region of BDF$_1$ mice on Day 0. Drug treatment at 3 dosage levels (0.62 decrements) began 3 days later and was continued until 40% or more of the mice at the top level were dead from toxicity (see "Materials and Methods"). All dosages less than or equal to an LD_{10} are listed. For controls, the MDD was 83; the time for the median tumor to reach 500 mg after trocar implant of 30- to 60-mg size fragments was 25 days (C value). There were no tumor-free survivors among the 10 control mice. The median exponential TD was 5.4 days.

Agent[a]	Dosage (mg/kg/dose)	Drug route and schedule	Drug deaths	% ILS[b] (excluding cures)	Tumor-free survivors on Day 157	Time for median tumor to reach 500 mg (excluding cures)	Median tumor growth delay (T-C in days)	Log$_{10}$ cell kill: Per dose	Log$_{10}$ cell kill: Gross	Log$_{10}$ cell kill: Net	Activity rating[c]
ADR	6.8	i.v., 3, 7, 11, 15	0/10	+87	8/10	84	60	0.84	3.36	2.68	++++
	4.2	i.v., 3, 7, 11, 15	0/10	+13	2/10[d]	53	28	0.39	1.56	0.90	++ — +++[e]
5-FU	65[f]	i.p., 3, 7, 11	0/10	+18	0/10	37	12.0	0.22	0.67	0.22	−
Cyclophosphamide	156	i.p., 3, 7, 11, 15, 19	1/10	+78	0/10	58	33.0	0.37	1.85	0.96	++
	96	i.p., 3, 7, 11, 15, 19	0/10	+73	0/10	38.5	13.5	0.15	0.75	−0.14	±
cis-DDPt	8	i.p., 3, 7	0/10	+25	2/10	20.5	20.5	0.57	1.14	0.82	++
	5	i.p., 3, 7	0/10	+18	1/10	38	13.0	0.36	0.73	0.50	+
BCNU	24	i.p., 3, 7	1/10	+19	1/10	34.5	9.5	0.28	0.53	0.31	−
	15	i.p., 3, 7	0/10	+25	2/10	38	14.0	0.39	0.78	0.56	+

[a] For list of abbreviations, see Table 4.
[b] % ILS, percentage of increase in host life span; T-C, tumor growth delay (median of group, excluding tumor-free survivors), evaluated at 500 mg to avoid complications of surface ulcerations and fluid production (common with Panc 03 at larger sizes).
[c] Where ++++ is highly active, and − is inactive.
[d] Five of 10 cures as of Day 87; 3 mice died of unknown causes but were tumor-free between Days 88 and 157.
[e] +++ activity rating reflects a more accurate level of activity because of the large number of cures (5 of 10 at Day 87).
[f] Lowest dosage used for 5-FU was 65 mg/kg/dose. The 2 higher dosage levels were excessively toxic.

Table 6

Comparison of the antitumor activity of Panc 02 and Panc 03 with other transplantable solid tumors of mice

Except for ROS, the activity ratings for all tumors listed, are based on the same criteria (see activity rating table in "Materials and Methods"). Activity ratings frequently varied by one rating unit from experiment to experiment. In all cases, the tumors were implanted s.c., and the agents were injected by another route (i.p., p.o., or i.v.). The activity ratings for ROS were based on partial regressions (>60% mass reduction) of advanced stage (0.5 to 2 g) tumors.

	Cyclophosphamide	PCNU[d]	cis-DDPt	ADR	Actinomycin D	PalmO-ara-C	5-FU	Procarbazine	Vincristine	Triazine antifol NSC 127755
Pan 02	−[b]	+	−	−	−	−	−	−	−	−
Pan 03	++	−	++	++++	NA[c]	NA	−	NA	NA	NA
Colon 38	++ — +++	−	+	++ — +++	+ — ++	++++	+	++	−	++++
Colon 51	+	+++ — ++++	++	±	−	−	−	−	−	−
Colon 26	++	++++	+++	− — +	−	−	++	−	−	+
Mamm 16/a	++ — +++	[illegible]	+	+++ — ++++	NA	++	++ — +++	NA	++	+
Fu IndOstSar	++++	[illegible]	+++	−	NA	−	−	NA	−	−
ROS	++++	[illegible]	++ — +++	+++ — ++++	++++	++ — +++	++ — +++	−	++ — +++	NA

[a] For list of abbreviations, see Table 4.
[b] − to +, no regressions; ++, 10 to 20% partial regressions; +++, 30 to 80% partial regressions; ++++, >80% partial regressions and 72% cures.
[c] NA, not available.
[d] BCNU [N,N'-bis(2-chloroethyl)-N-nitrosourea].

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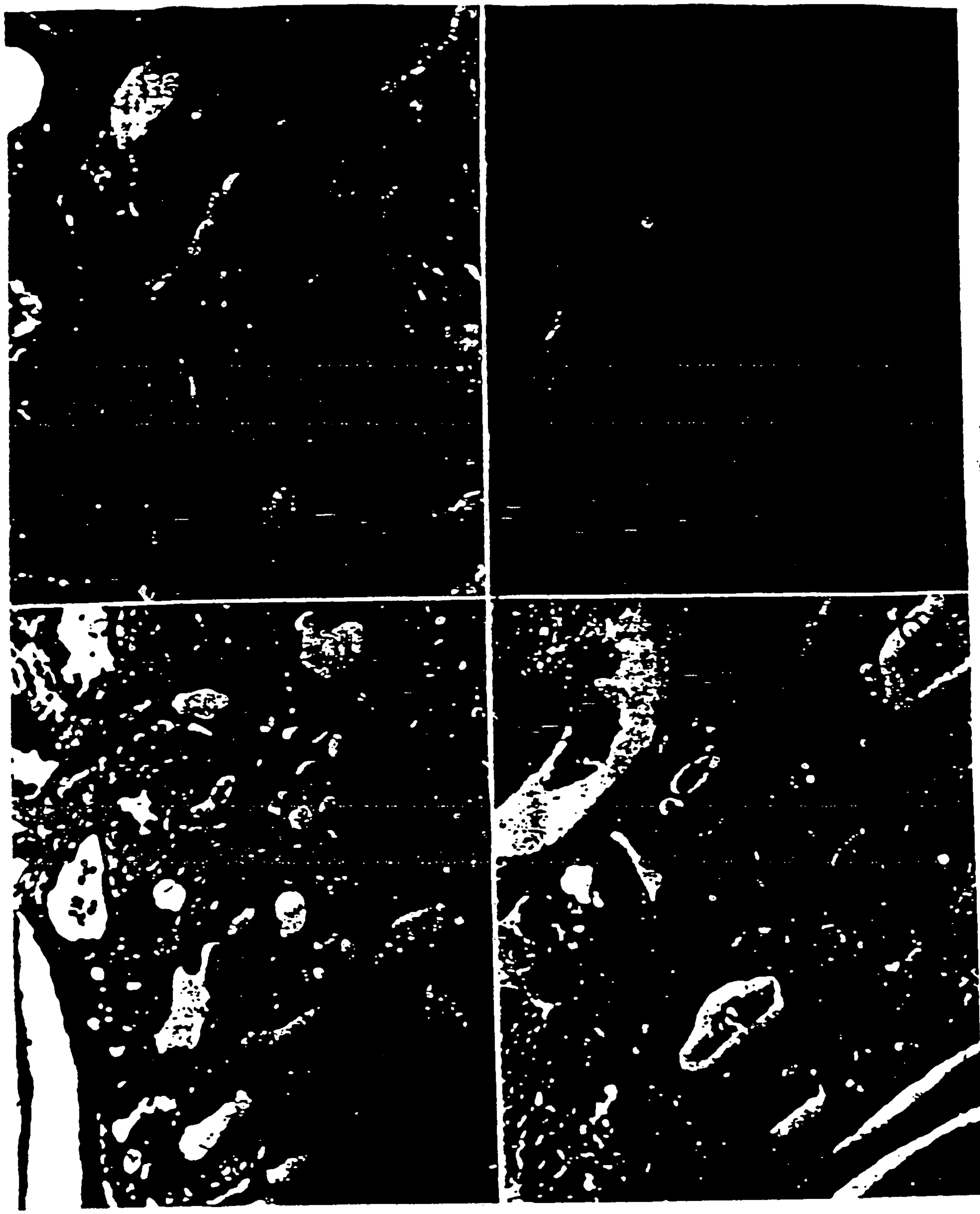

Fig. 1. Pancreatic Tumor 02, original tumor. Several moderately well-differentiated glands are present. H&E × 40 (original magnification).
Fig. 2. Pancreatic Tumor 02, 54th transplant generation (after cell culture passage). The tumor is composed of solid sheets of epithelial cells with limited glandular formation. H&E × 40 (original magnification).
Fig. 3. Pancreatic Tumor 02, 54th transplant generation. The epithelial cells have a uniform appearance. H&E × 100 (original magnification).
Fig. 4. Pancreatic Tumor 03, original tumor. A well-differentiated adenocarcinoma. H&E × 40 (original magnification).

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